Host Response to Botulinum Neurotoxins for Developing Diagnostics and Antidotes

(DoD/Army Contract No. W911NF-06-1-0095)

Final Report

by

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Date: September 18, 2009

Report Documentation Page		*Form Approved* *OMB No. 0704-0188*

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1. REPORT DATE **18 SEP 2009**	2. REPORT TYPE	3. DATES COVERED **00-00-2009 to 00-00-2009**
4. TITLE AND SUBTITLE **Host Response to Botulinum Neurotoxins for Developing Diagnostics and Antidotes**		5a. CONTRACT NUMBER **W911NF-06-1-0095**
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Botulinum Research Center,University of Massachusetts Dartmouth,285 Old Westport Road,Dartmouth,MA,02747**		8. PERFORMING ORGANIZATION REPORT NUMBER **; 49439-CH.1**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) **U.S. Army Research Office, P.O. Box 12211, Research Triangle Park, NC, 27709-2211**		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S) **49439-CH.1**
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release; distribution unlimited**		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT		
15. SUBJECT TERMS		

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**65**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

The goals of the project are to investigate host-response to the exposure of botulinum neurotoxin (BoNT) and its neurotoxin associated proteins (NAPs) by examining immunological effects of different components of BoNT complex, using in vitro lymphocytic cell cultures as well as an animal model so that we can understand, both the immune response as well as characterize damage to the immune system of botulism patients, by identifying the most critical components of BoNT/A complex involved in the binding to the intestinal wall for the translocation across its mucosal layer, and to identify and isolate a protein receptor(s) for BoNT/A or its NAPs, and examine its binding and involvement in the transcytosis/penetration process.

The immune response and other cellular responses are targeted for presymptomatic diagnostics, whereas identification of the protein receptor(s) of BoNT or NAPs and also critical components NAPs in the binding and translocation of the BoNT would provide target for the development of antidotes against botulism.

The project started in May 2006, and we have just begun to prepare reagents and collect preliminary data. Since BoNT is highly regulated agent, and this project involves close collaboration with Drs. Frank Lebeda, Martha Hale, and Harry Hines of USAMRIID, we have produced two recombinant proteins – a mutant BoNT/A that lacks the endopeptidase activity, and the hemagglutinin 33 (Hn-33), the most abundant component of NAPs.

In accordance with a new hypothesis proposed from our group in 2005, we demonstrated that the Hn-33 binds directly to the nerve cells even at low concentration of 30 nM to the surface of the SH-SY5Y cells (a human neuroblastoma cell line) as observed under confocal microscope. As shown in **Figure 1**, Hn-33 was only attached to the surface when incubated for less than 10 min, but was clearly observed inside the plasma membrane after incubation of Hn-33 with the cells for 15 min. These results establish a time dependent process in the binding and entry of Hn-33.
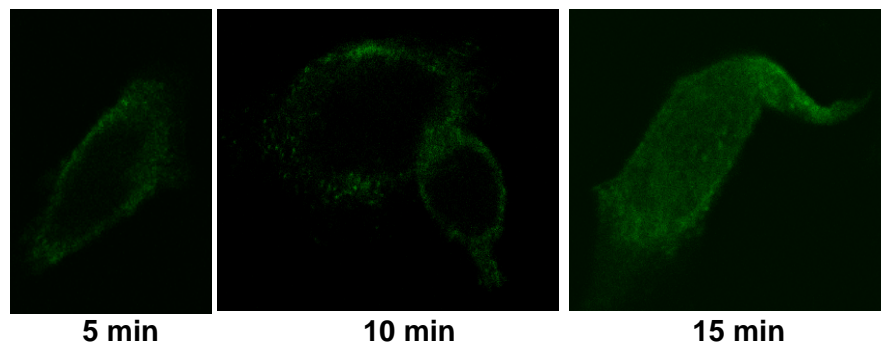

| **5 min** | **10 min** | **15 min** |

Figure 1. Binding and internalization of Hn-33 to SH-SY5Y cells at different incubation periods of time. Hn-33 at 30 nM in fresh culture medium was incubated with SH-SY5Y cells for 5 min, then removed the medium and rinsed the cells with fresh culture medium. The cells were fixed after the cells were incubated in fresh medium at 37°C for 5, 10, 15 min, respectively. Rabbit anti-Hn-33 antibody was used as primary antibody and anti-rabbit IgG FITC-conjugate was used as secondary antibody.

Initial experiments to evaluate Hn-33 as a superantigen candidate was carried out with cultured human peripheral blood mononuclear cells (hPB) after isolating them by Ficoll-

hypaque density gradient centrifugation (25). The "buffy coat" was washed in Hank's balanced salt solution and then cultured in RPMI 1640 media supplemented with 10% heat inactivated fetal calf serum for three to five days in media containing various concentrations of Hn33 or no Hn33 (untreated control). Phenotype of cells was determined by fluorescence activated flow cytometry (FACS) using appropriate IgG recognizing determinants on specific cell types. Cell types included CD3+CD4+ (Th), CD3+CD8+ (CTL), CD56+CD3- (NK), CD56+CD3+(NKT), CD11c+ABC or CD11c+DR (dendritic cells with hla class I or hla class II), and CD11c-ABC or CD11c- DR (Non dendritic cells) and antigen presenting cells.

While it was clear that the Hn-33 is not a superantigen, it clearly activated T helper cells as well as cytotoxic lymphocytes (**Fig. 2**). Our preliminary observations also suggest Hn-33 activitation of dendritic cells but further confirmation is awaited. These initial observations clearly suggest that the immune response to BoNT/A NAPs is robust and it may be possible to design ways to use the immune response for diagnostics.

We have also started culturing epithelial cells to examine binding and cellular response to BoNT/A and it NAPs. We are have labeled Hn-33 with Alexa red which will be examined by Dr. Hale at USAMRIID for its translocation across the monolayer of epithelial cells. Exposure of ephithelial cells to Hn-33 and DrBoNT/A is being examined by Dr. Harry Hines of USAMRIID using mass spectroscopy.
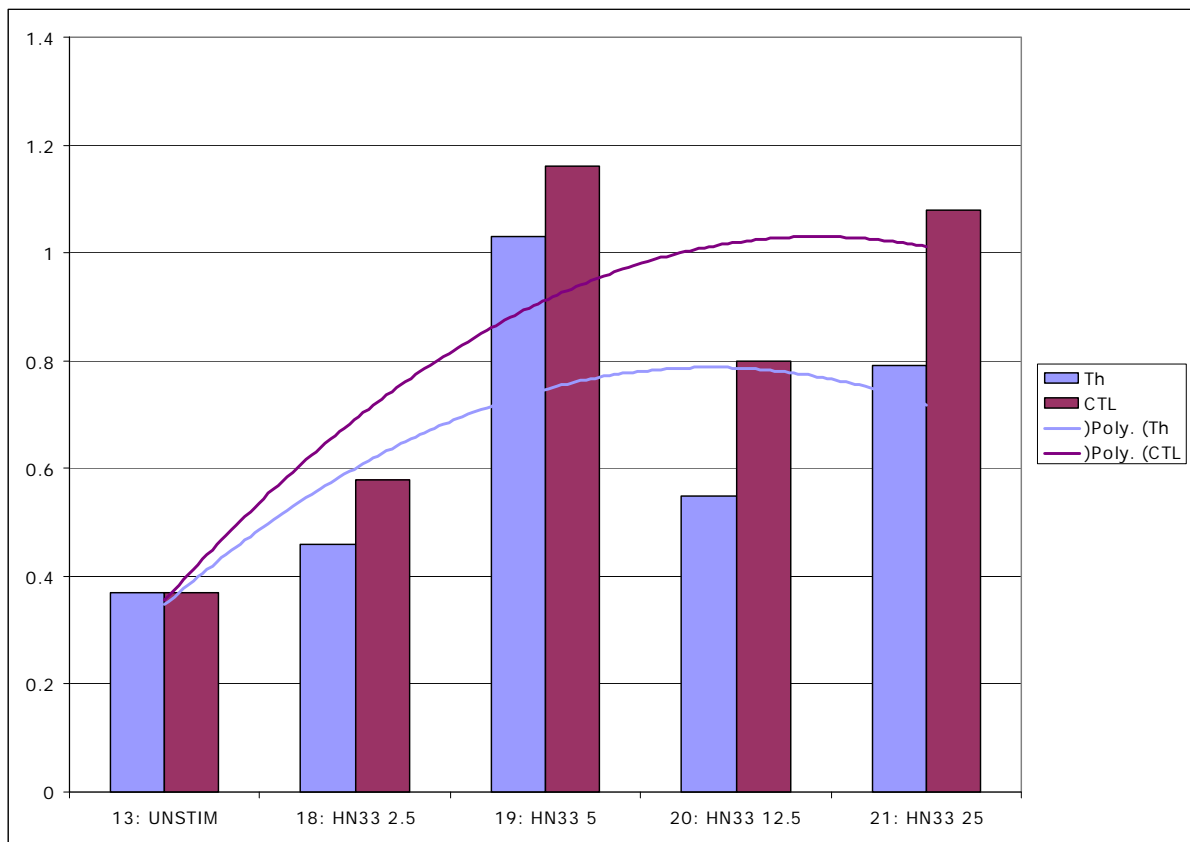

Figure 2. Th and CTL response to different concentrations of Hn-33 exposed during 3-day culture of cells.

Immunological characterization of the Subunits of Type A Botulinum Neurotoxin and Different Components of its Associated Proteins

INTRODUCTION

Botulinum neurotoxins (BoNTs) are a group of large proteins produced by *Clostridium botulinum* in seven immunologically distinct serotypes named A to G. BoNTs being the causative agents of the most dreaded food poisoning disease, botulism, are the most potent toxins known to human kind (Singh, 2000). Interestingly, however, BoNT also remarkably serves as a powerful tool to treat a myriad of neuromuscular disorders, and in cosmetic applications (Klien, 2004; Bhidayasiri and Troung, 2005).

BoNTs are produced as ~ 150 kDa nontoxic single chain proteins that are activated by proteolytic cleavage to a dichain structure consisting of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) linked through a disulfide bond. To produce its toxic effect, the toxin progresses through a series of well defined steps that includes binding to receptors on the surface of cholinergic nerve endings mediated by the C-terminus of heavy chain, internalization via receptor mediated endocytosis, penetrating the endosome by pH induced translocation, and finally acting enzymatically in the cytosol to block neurotransmitter release. BoNTs possess Zn^{2+} endopeptidase activity against a select group of neuronal proteins involved in the exocytosis process, causing the blockage of acetylcholine release at the neuromuscular junction (Montecucco and Schiavo, 1995; Dressler and Saberi, 2005).

The most common source of botulism is by ingestion of food contaminated with spores of *C. botulinum*, preserved under anaerobic conditions that favor germination of spores, and secretion of the neurotoxin (Chen et al., 1998). BoNTs are secreted from the bacterium along with a group of neurotoxin associated proteins (NAPs) in the form of a complex. The size of the complex varies with serotypes, but all the serotypes of botulinum neurotoxin complex contain 1-7 additional associated proteins (Sakaguchi, 1983; Cai et al., 1999). A close functional relationship between BoNT and NAPs is strongly indicated by clustering of genes for BoNT, NAPs and a regulatory gene botR (Cai et al., 1999). NAPs have been known to contribute significantly to the oral toxicity of BoNTs (Li et al., 1998). NAPs also have been recently shown to play a critical role in enhancing the endopeptidase activity of the neurotoxin (Sharma and Singh, 2004; Kukreja and Singh, 2007). Because of the extreme toxicity and stability of BoNT in the presence of NAPs, BoNT complexes are on the top of the list of biological warfare agents (Sharma and Singh, 2004). Ironically, it is the complex form of the BoNT along with NAPs that is most commonly used as therapeutic agent to treat several neuromuscular disorders and in cosmetic applications (Shukla and Sharma, 2005).

Specific interactions between the neurotoxin and NAPs in botulinum complex seem to protect the neurotoxin from the acidity and proteases in the GI tract and in the absorption of the toxin in the gut wall (Fu et al., 1998; Fujinaga et al., 1997). At the same time, such protection could interfere with the interaction of an antibody with the neurotoxin when present in the complex form. Therefore, it is important not only to analyze the interference of the NAPs in the detection of the neurotoxin, but also to characterize the immunological properties of the associated proteins themselves. So far, studies have been conducted only on the effect of BoNT at the nerve cell in blocking the release of neurotransmitters to cause flaccid paralysis. BoNT and NAPs could have an

effect on other vital systems, in addition to paralysis. Understanding the effect of BoNT and NAPs on the immune system is of particular concern. Thus immunological characterization of BoNT and its associated proteins is critical for not only designing detection systems for this class A bioterror agent, but also for developing more efficient approaches for vaccine development against botulism, and to better understand their immune response in the host.

In this study we have investigated the immunochemical reactivities of BoNT/A in its pure and complex forms, and its associated proteins, to polyclonal antibodies raised against the toxoids of the neurotoxin complex, pure neurotoxin, and its constituent light and heavy chains, and Hn-33 using enzyme-linked immunosorbent assay.

Results indicate that the neurotoxin is accessible to antibodies even in the complex form, but such accessibility is hindered by the presence of NAPs. In addition, antibodies raised against the whole complex have higher reactivity to the complex and NAPs when compared to the purified neurotoxin, suggesting stronger immunogenicity of the NAPs over that of the purified neurotoxin. Higher immunogenicity of the neurotoxin complex is primarily due to the presence of Hn-33. BoNT/AC, NAPs and Hn-33 have a significantly higher potential to induce host response.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), o-phenylenediamine dihydrochloride (OPD), 3% H_2O_2, and polyoxyehtylene sorbitan monolaurate (Tween-20), and alkaline phosphatase conjugated goat-anti rabbit IgG were obtained from Sigma Chemical Co., St. Louis, MO). Phosphate-buffered saline (0.01 M PBS, pH 7.4), Tris-buffered saline (0.01 M Tris, pH 7.4), and phosphate-citrate buffer (PCB, pH 5.0) were prepared in deionized distilled H_2O. All chemicals were of the highest grade commercially available.

Preparation of botulinum neurotoxin type A in its complex and pure forms, recombinant Hn-33, recombinant BoNT/ALC, BoNT/A HC and their toxoids.

Type A neurotoxin (BoNT/A) and its complex (BoNT/AC) were prepared according to previously established procedures (DasGupta and Satyamoorthy, 1984). During the purification of the neurotoxin, we obtained a pool containing mostly NAPs with residual toxin. The pool was dialyzed against 0.02M Na_2HPO_4,-NaH_2PO_4 buffer, pH 7.0, and loaded on a SP-Sephadex C-50 column that was equilibrated with the pH 7.0 buffer. Recovery of the purified NAPs eluted from the SP-Sephadex column during the column wash was ~95%. The residual neurotoxin remained bound to the SP-Sephadex column and needed a linear gradient of increasing Na^+ for its elution.

Recombinant Hn-33 and LC were prepared as described previously (Zhou et al., 2007; Li and Singh, 1999). BoNT/A heavy chain was isolated from detoxified recombinant BoNT/A, that had been produced in our lab (Yang et al., 2008), by nicking and subsequent separation of the heavy chain from the detoxified holotoxin. Detoxified BoNT/A is recombinant full length botulinum type A with mutation in its two active site residues E224A/E262A, that has been successfully cloned, expressed in *E. coli* as a His-tagged protein, and purified from a Ni^{2+}-affinity column in our lab. The purified protein lacks the endopeptidase activity involved in the toxic action of the BoNT, leading to its detoxification. For separation of BoNT/A heavy chain, purified detoxified recombinant in 0.05 M sodium phosphate, 0.3 M sodium chloride, pH 7.5 was incubated with trypsin (200:1 protein:enzyme ratio) for 60 minutes at room temperature. The reaction was

stopped by the addition of trypsin inhibitor. The light and heavy chains were separated according to the previously established procedure (Sathyamoorthy and DasGupta, 1985). Briefly, the nicked deactivated toxin was dialyzed against 0.02 M sodium borate, 0.04 M sodium phosphate, pH 8.4 and applied to a QAE-Sephadex A50 column equilibrated with the same buffer. The material was reduced first with the aforementioned buffer containing 0.01 M DTT and then incubated with the same buffer containing 0.1 M DTT and 2 M urea overnight. Chromatography was resumed with application of buffer containing 0.01 M DTT and 2 M urea which eluted the light chain. The bound heavy chain was eluted with 0.01 M DTT, 2 M urea and 0.2 M sodium chloride.

All proteins used to immunize rabbits were prepared as toxoids. For preparation of toxoids, BoNT/AC, pure BoNT/A, Hn-33, BoNT/A HC, and BoNT/A LC were precipitated with ammonium sulfate and the precipitant was dissolved in 0.1 M sodium phosphate buffer (NaPB) containing 50 mM NaCl, pH 7.4. The toxin was inactivated by dialyzing it against buffer containing 0.5 % (wt/vol) of formaldehyde at 4 ºC for 6 days. Formaldehyde containing buffer was replaced everyday with fresh solution. Dialysis was continued for the next 7 days at room temperature with a single fresh buffer change every day. Formaldehyde was removed by dialyzing the proteins against 100 mM NaPB containing 50 mM NaCl, pH 7.4 for two days with several fresh buffer changes.

Immunization of rabbits with BoNT/AC, BoNT/A, BoNT/A LC, BoNT/A HC, and Hn-33

Rabbits were immunized with formaldehyde inactivated proteins (toxoids). Rabbit antisera against BoNT/AC, BoNT/A, Hn-33, BoNT/A LC and BoNT/A HC were prepared by Biodesign International Inc. on a contract basis. Briefly, rabbits were primed with antigens (400 µg, 1ml) subcutaneously along with complete Freund's adjuvant (0.5 ml) on day 0 at multiple sites, followed by booster doses on days 21, 42, and 65 with antigens (200 µg, 0.5 ml) in incomplete Freund's adjuvant (0.5 ml). The animals were initially bled on day 51 to test the antibody titer and from day 74 onwards anti-rabbit sera was collected at regular intervals of 14 days and terminally bled at ~ day 140 .

ELISA

Flat-bottom 96 well microtiter plates were coated with 1 µg/ml of antigens that were not treated with formaldehyde (BoNT/AC, BoNT/A Hn-33, NAPs, BoNT/A LC, BoNT/A HC) dissolved in 0.01 M phosphate buffered saline (PBS) at pH 7.4 and allowed to adhere overnight at 4 ºC. To remove any unbound antigen, plates were washed thrice with 0.01 M PBS, pH 7.4 containing 0.05 % Tween-20 (PBST) and subsequently blocked with 3% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.4 and incubated for 1 h at 37 ºC. Following a washing step as outlined above, plates were incubated for 1h at 37 ºC with serial dilutions of the sera in 3% BSA. As controls, column 1 (no antigen) was incubated with the lowest dilution of the serum while column 2 (no primary antibody) was incubated with 3% BSA. Following a washing step, plates were incubated with peroxidase-labeled anti-rabbit IgG secondary antibody (1:18,000 dilution), for 1 h at 37 ºC. Plates were washed four times with PBST and then incubated with 100 µl of a substrate solution containing 0.04% OPD (o-phenylenediamine dihydrochloride) and 0.012% hydrogen peroxide in 0.15M citrate-phosphate buffer, pH 5.0 for 20 min at room temperature (25 ºC). The reaction was subsequently quenched with 50 µl of 2M sulfuric acid and the absorbance was measured at 490 nm. The antibody titer of the sera was

measured by plotting absorbance vs. serum dilution using the mean and standard deviation for each triplicate set.

SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of BoNT/AC (non-formaldehyde treated) was run on a 4-20% gel using the Mini Protean III system from BioRad at a constant voltage of 200 V at room temperature. The protein bands were visualized by Coomassie blue staining. The gel was scanned on a GEL Logic 100 Imager system, analyzed and quantified using the Kodak Image analysis software (Eastman Kodak Co., Rochester, NY).

Western blot analysis

Western blot analysis was carried out using polyclonal rabbit anti-BoNT/AC IgG (BB Tech, Dartmouth, MA). SDS-PAGE was run using a Mini Protean III system from BioRad at a constant voltage of 200 V at room temperature (25C) using a 4-20% gradient gel. The proteins were transferred to Immun-Blot PVDF membrane (BioRad, Hercules, CA) using Mini TransBlot system from BioRad. Membrane was incubated in blocking buffer consisting of 3% BSA in 0.01 M TBS, pH 7.4 with gentle shaking for 2 h at room temperature. Membrane was washed with 0.01 M TBS, pH 7.4 containing 0.05% Tween-20 (TBST) thrice for 5 min duration. Membrane was incubated with rabbit anti-BoNT/AC IgG (1:3000 dilution) in blocking buffer for 1 h at room temperature with gentle shaking. Following a washing step as outlined above, the membrane was incubated with goat anti-rabbit IgG alkaline phosphatase antibody (BioRad, Hercules, CA) in blocking buffer for 1 h at room temperature with gentle shaking. Colorimetric detection was carried out using 5-bromo-4-chloro-3'-indoylphosphate p-toluidene salt (BCIP) and nitro-blue tetrazolium chloride (NBT) (BioRad) as substrate, after the membrane was washed thrice with TBST. The membrane was scanned on a GEL Logic 100 Imager system and analyzed and quantified using the Kodak Image analysis software (Eastman Kodak Co., Rochester, NY).

Serum neutralization assay:

The functional antibody titers of BoNT/AC and pure BoNT/A were tested in their capacity to neutralize pure BoNT/A and BoNT/AC in a mouse model, which is the standard assay used by the WHO (Jones et al., 2006). Anti BoNT/AC and anti-BoNT/A sera collected from immunized rabbits were either used as the whole sera or after dilution with PBS containing 0.2% BSA, and combined with 1 or 10 mouse LD_{50s} of BoNT/A. The antisera and toxin mixtures was incubated at room temperature for 1 h before being administered to Swiss Webster mice (0.5 ml, intraperitoneally, i.p). The mice were monitored for 96 h to assess the residual toxicity of various mixtures and survival was scored.

N-terminal sequencing of P-250 protein

The N-terminal sequencing of P-250 protein was carried out by running BoNT/AC on SDS-PAGE as described above, followed by transferring the protein from the gel onto a Sequi-Blot PVDF membrane (Bio-Rad, Hercules, CA) using the semi dry TransBlot system from BioRad (Hercules, CA). The protein was allowed to transfer until the voltage dropped from 200 V to 80 V. The Sequi-Blot PVDF membrane was first stained with Coomassie blue and then destained until a clear background was obtained. The P-250 protein was sequenced using an Applied Biosystems Precise Sequencer at the Baylor College of Medicine, Houston, TX.

Sequence Analysis of P-250 protein

Based on the N-terminal sequence of P-250, we used the codon usage table of *Clostridium botulinum* Hall-A (strain ATCC 3502) to predict the nucleotide sequence from amino acids back translation program that was acquired from Entelechon company by using their "BacktranslationToolV2.0" [http://www.entelechon.com/eng/backtranslation.com].

The genomic sequence of *Clostridium botulinum* type A (ATCC3502) was obtained from the Sanger Institute (http://www.sanger.ac.uk/Projects/Microbes). The backtranslated P-250 nucleotide sequence was used for the sequence search and alignment with 7 microbial genome databases in NCBI GenBank by using BLASTN and TBLASTN (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). The 7 microbial genome databases comprise of *Clostridium acetobutylicum* ATCC 824, *Clostridium botulinum* type-A ATCC3502, *Clostridium difficile* 630, *Clostridium perfringens* ATCC13124, *Clostridium perfringens str.* 13, *Clostridium tetani* E88, *Clostridium thermocellum* ATCC27405.

The parameters for running TBLASTN were set as "Low Complexity Filter is checked, Expect value= 1.0". Search for the theoretical ORFs which included the P-250 nucleotide sequence was carried out using the Hidden Markov Model GeneMark software (http://opal.biology.gatech.edu/GeneMark). The search area covered the total 48KB (24kb downstream and 24kb upstream to the hit sequence) genomic sequence area.

GeneMark is a trademark that includes a family of gene prediction programs provided by Mark Borodovsky's Bioinformatics Group at the Georgia Institute of Technology, Atlanta, Georgia (Lukashin and Borodovsky, 1998; Besemer and Borodovsky, 1999). The putative P-250 ORF protein was analyzing by using the "ExPASy Proteomics Tools" (http://ca.expasy.org/tools) Category "Protein identification and characterization tools" (Gill and von Hippel, 1989; Altschul et al., 1990).

RESULTS AND DISCUSSION
Immunogenicity of botulinum neurotoxins and its associated proteins

Immunoreactivity of sera isolated from rabbits immunized with BoNT/AC, pure BoNT/A, BoNT/A LC, BoNT/A HC, NAPs, and recombinant Hn-33 were analyzed by ELISA.

In order to compare the immunoreactivity of BoNT/AC to BoNT/A and to the neurotoxin associated proteins, interaction of α-BoNT/AC sera with BoNT/AC (non-formaldehyde treated), pure BoNT/A, Hn-33, and NAPs was examined. As shown in Figure 1, it is clear that antibodies raised against the entire complex have a much stronger reactivity to the complex than to the neurotoxin. Results showed that rabbit α-BoNT/AC sera titers to BoNT/A antigen was detected but with 60- fold lower titer than for BoNT/AC. However, significant reactivity of anti-complex sera to the purified neurotoxin was still observed indicating that anti-complex sera contains a substantial amount of antibodies that can interact with the neurotoxin. This suggests that the use of antibodies against the purified neurotoxin may not be the best system for the detection of

botulinum neurotoxin and that the detection of the neurotoxin complex may be more sensitive allowing for the detection of the neurotoxin at a much lower concentration. Detection of botulinum neurotoxin indirectly through the detection of the neurotoxin complex may have a significant value in ensuring food and environmental safety.

It was also observed that the relative reactivity of rabbit α-BoNT/AC sera to neurotoxin associated proteins and to Hn-33 was close to BoNT/AC and its reactivity to Hn-33 and NAPs was 35-fold higher than that of pure BoNT/A antigen (Figure 1). This was also confirmed by the SDS-PAGE and Western blot analysis of BoNT/AC (Figure 2). SDS-PAGE of BoNT/AC (Figure 2A, lane 2) exhibited 8 protein bands corresponding to the neurotoxin and a group of associated proteins. Based on the protein yield of the neurotoxin and associated proteins in the complex, it has been estimated that BoNT/AC has about 25% neurotoxin and 75% associated proteins (Fu et al., 1998; Sharma et al., 2003). For BoNT/AC the protein bands correspond to 145 kDa (BoNT/A), 120 kDa NBP (neurotoxin binding protein), 53 kDa (NAP-53), 33 kDa (Hn-33), 30 kDa (Hn-30), 20 kDa (NAP-20), 17 kDa (NAP-17) and 14 kDa (NAP-14). The assignments of bands observed on SDS-PAGE gel are based on previous biochemical and genetic analysis of BoNT/A in its complex and pure forms and of its associated proteins (Cai et al., 1999; Fu et al., 1998; Kukreja and Singh, 2007). For NAPs, the protein bands correspond to 120 kDa NBP (9%), 53 kDa NAP-53 (16%), 33 kDa Hn-33 (25%), 30 kDa Hn-30 (9%), 20 kDa NAP-20 (9%), 17 kDa NAP-17 (5%), and 14 kDa NAP-14 (8%), which is similar to previously published results (Fu et al., 1998).

Western blot analysis of BoNT/AC (Figure 2B) shows that Hn-33 is most immunogenic of all the NAPs in the complex and the neurotoxin shows less immunogenicity. Interestingly, a protein band at ~250 kDa showed higher immunogenicity in comparison to the neurotoxin (Figure 2B). Presence of this protein in the neurotoxin complex may in part be responsible for higher immunogenicity of the complex.

Neutralizing capacity of rabbit anti-BoNT/A and anti-BoNT/AC antibodies

The neutralizing capacity of rabbit anti-BoNT/AC and anti-BoNT/A sera to purified BoNT/A and BoNT/AC was determined using the mouse bioassay. The toxicity of pure BoNT/A and BoNT/AC incubated with pre-immune serum was equivalent to that of the two toxins incubated in physiological saline. By contrast, the undiluted anti-rabbit sera of BoNT/A or BoNT/AC completely neutralized 10 lethal doses of pure BoNT/A (Figure 3A) or BoNT/AC (data not shown).

In addition, both anti-rabbit sera were tested at the 50% titer of anti-BoNT/AC sera [~1:190000, Figure 5] diluted with physiological saline and 1% BSA. Animals that had been immunized with BoNT/AC completely neutralized pure BoNT/A activity (100% protection; 1LD50 dose; Figure 3B), whereas serum from animals immunized with pure BoNT/A did not seem to neutralize pure BoNT/A activity completely (Figure 3B). It offered about 15% protection at $1LD_{50}$ dose (Figure 3A), indicating that anti-BoNT/AC serum elicits higher protection in comparison to anti-BoNT/A serum. Anti-BoNT/A sera was also tested at its 50% titer [~1:16000, Figure 5] diluted with physiological saline and 1% BSA. It offered about 60% protection at $1LD_{50}$ dose (data not shown), again suggesting that anti-BoNT/AC serum elicits higher protection in mice in comparison to anti-BoNT/A serum.

These observations suggest that antibodies against the toxin were present more in the anti-sera from rabbits immunized with BoNT/AC compared to the antiserum from rabbits immunized with pure BoNT/A. This conclusion is consistent with the higher binding of BoNT/AC antibodies to BoNT/AC reinforcing our observation of higher immunogenicity of BoNT/AC.

Discovery of a highly immunogenic new protein

In an initial set of experiments, we cut out the protein band observed at ~250 kDa, and carried out N-terminal amino acid sequencing for the first 15 amino acid residues. The sequencing result, LLDSSESITLDKTSM, was unique, and matches with a single ORF in *Clostridium botulinum* type Hall strain (ATCC3502) genome (NCBI accession number: YP_001254150) (Lindo et al., 2004). The putative ORF site encompassing the peptide identified by the peptide sequencing is located at the genome map position 1,794,882 of ATCC 3502 Hall-A, in contrast to BoNT gene being at position 909,708. Therefore, it is clear that P-250 does not belong to the BoNT gene cluster and is located 885,114 bp downstream (Figure 4A). Interestingly, the gene corresponding to the peptide sequence of P-250 has the start codon methionine at 190 amino residues upstream to the N-terminal sequence we obtained (Figure 4B), suggesting that the polypeptide gets digested either during the bacterial growth or during the purification process of BoNT/AC. The polypeptide segment corresponding to the downstream of the peptide sequence identified adds up to a total of 169 amino acid residues, including the N-terminal 15 amino acid sequenced (Figure 4B). The molecular size of this segment corresponds to only 18.74 kDa. Therefore the P-250 band is likely to be a SDS-resistant oligomer of this 18.74 kDa segment, consisting of about 16 monomers. This conclusion needs to be confirmed with further experiments. Isolation and characterization of this protein in the botulinum type A complex is currently under investigation in our laboratory.

BLAST P analysis of the newly identified protein sequence consisting of the protein segment beginning with the peptide identified by our N-terminal peptide sequence analysis showed over 97% sequence homology with bacterial Ig-like proteins, Intimins, found on the surface of bacterial cells or phage particles. The polypeptide has sequence homology ranging between 64 to 97% with *Clostridium* Type B, F, and A3, *Clostridum beijerinckii,* and Bacillus (Table 1) proteins which have been classified as cell adhesive proteins. A sequence homology analysis encompassing the whole protein that begins with the ORF starting amino acid showed significantly lower homology. The segment showing the maximum homology matches with the most conserved Bacterial Ig-like domain (Big_2 domain). Big_2 domains are conserved domains of cell adhesion proteins found in many bacterial cells (Kelly et al., 1999).   Since these proteins are present on the surface of the bacterial cell, it is likely that P-250 protein identified in BoNT/AC is present on the surface of the *Clostridium botulinum* cells, and possibly gets attached to BoNT/AC during the latter's release from the bacterial cells. Further experiments are needed to determine that P-250 protein is indeed associated with BoNT/AC and is not just a minor contaminant that is co-purified with the complex Although not reported previously, a protein of similar size can be seen on SDS-PAGE gels of not only other BoNT/AC preparations (Inoue et al., 1996) but also types C and D BoNT complexes (Inoue et al., 1999). Relevance of the immunogenicity of P-250 protein needs to be further examined both for detection of BoNT agents and also for their

immunogenic response in therapeutic preparations commercially being used against several neuromuscular disorders.

Relative immunoreactivity of Hn-33

Higher reactivity of rabbit α-BoNT/AC sera to the neurotoxin associated proteins and to Hn-33 could result from the abundance of associated proteins in the complex or due to higher immunogenicity of these proteins. Relative immunogenicity of BoNT and NAPs was investigated by testing the binding of BoNT/AC, Hn-33, purified BoNT/A, and BoNT/A light and heavy chains with their respective antisera. It was observed that sera raised against Hn-33 binds to Hn-33 three times more effectively than anti-neurotoxin to the neurotoxin (Figure 5). It is notable that although the composition of Hn-33 and BoNT/A in the complex are identical (~25%; Sharma and Singh, 2000), the two antigens generated different immune responses. These results clearly points towards stronger immunogenic properties of Hn-33. Inspite of being a smaller protein in comparison to the BoNT/A (33 kDa v/s 150 kDa), Hn-33 exhibited stronger immunogenicity. Since Hn-33 forms the largest fraction of NAPs in the botulinum complex, this observation indicates that Hn-33 accounts for most of immune response of the complex. It was also observed that BoNT/A LC was 2-fold more immunogenic than BoNT/A HC. This observation is at variance with the observation of Chen et al., 1997, whose results with monoclonal single chain variable fragments have suggested that heavy chain may be more immunogenic based on how many scFv reacted to the heavy chain domain vs. the light chain domain.

Medical implications of immunogenicity of different components of BoNT/AC

Although botulinum neurotoxins are being beneficially employed for the treatment of neuromuscular disorders, they have also been given high priority for the development of vaccines and therapies to prevent intoxication. Botulism can be prevented by administration of neutralizing antibodies or vaccination. Currently available vaccines are composed of chemically inactivated crude isolates of BoNTs that are produced in *C. botulinum* and have several limitations including efficacy and accessibility. Development of more efficient vaccines for botulism is warranted. Our results of Hn-33 and other NAPs exhibiting strong immunogenicity suggest that it may be possible to employ them as adjuvants for development of vaccines against botulism to enhance their efficacy. NAPs have previously been shown to demonstrate adjuvant activity in BoNT/B by increasing anti BoNT/B antibody production in mice (Lee et al., 2005).

Botulinum in its complex form is commonly used in therapeutic and cosmetic formulations (Wenzel, 2004). Development of resistance to botulinum therapy is an important clinical issue and antibodies raised against the toxin are responsible for most cases of resistance by development of neutralizing antibodies and secondary non-responsiveness to treatment (Atassai, 2004; Brin, 1997). This issue becomes more important with higher and repeated dosing. Our results of pure neurotoxin generating significantly less antibody response as compared to botulinum complex, suggest that pure neurotoxin may be less likely to induce antibodies which could lead to non-responsiveness. Botulinum toxin type A in its pure form free of associated proteins has recently been shown to be successful in the treatment of cervical dystonia (Benecke et al., 2005).

Recent reports have suggested that there are substantial systemic adverse reactions and respiratory compromise as a result of the use of types A and B botulinum

neurotoxin complex based therapeutic and cosmetic products (Botox[TM] and Myrobloc[TM]), leading United States Food and Drug Administration to issue medical advisory on their safety (http://www.fda.gov/cder/drug/early_comm/botulinium_toxins.htm). Immuno-reactivity of different components of BoNT/A complex may lead to examination of the role of these components in any possible adverse immune reactions.

Immuno-accessibility of the neurotoxin in botulinum neurotoxin complex

In order to investigate the accessibility of the neurotoxin within the complex, the immunoreactivity of sera against the pure neurotoxin to pure BoNT/A was investigated in the BoNT/A complex and pure forms and in the light and heavy chains of BoNT/A. ELISA binding curves of BoNT/AC and BoNT/A with antibodies raised against BoNT/A indicate that BoNT/A in its purified and complex forms reacts equally to the antibodies raised against the pure BoNT/A, but this reaction is about 2.5 fold higher compared to the immunoreaction to BoNT/A light and heavy chains (Figure 6). This observation suggests that α-BoNT/A can recognize similar epitopes in the pure neurotoxin or in the neurotoxin within the complex, but separation of the chains results in loss of some of the epitopes, presumably as a result of conformational changes which occur following the chain separation (Singh and DasGupta, 1989).

Comparison of the immunoreactivity of pure BoNT/A to antibodies raised against BoNT/AC (~1:3000, Figure 1) with the immunoreactivity of BoNT/AC to antibodies raised against pure BoNT/A (~1:16000, Figure 6) shows a substantial difference in the two reactivities (over 5-fold). At first it may simply be suggested that since BoNT/A is about 25% of the BoNT/A complex protein, given the same amount of protein injected for BoNT/AC toxoid and pure BoNT/A toxoid into rabbits, it will just be the effect of the amount of protein. However, the same could be true for Hn-33 which is also about 25% of BoNT/AC protein (Fu et al., 1998), but is not. Assuming that the lower immunoreactivity of BoNT/A in the complex is not due to the amount of antigen injected, the observation will reflect that the neurotoxin's accessibility may be playing a role in lower immunogenicity of BoNT/AC toxoid prepared from formalin treatment which causes crosslinking and freezing of the structure. BoNT/A in its complex structure appears to be in a flexible accessibility, which allows its accessibility to anti-BoNT/A antibodies, but once the structure is fixed with formalin crosslinking, only portion of it is available as antigen, perhaps leading to its lower immunogenicity. In contrast, Hn-33 in fact shows higher immunogenicity in a BoNT/A complex that after its purification. Hn-33 being on the surface of the complex (Hasegawa et al., 2007), but perhaps under either a different conformation or as a result of adjuvant effect of some of the NAPs in the complex. Further investigation is needed to address some of these questions.

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FIGURE LEGENDS

FIGURE 1. ELISA binding curves of antibodies raised against BoNT/AC to BoNT/AC, NAPs, Hn-33, and to BoNT/A.

FIGURE 2. A. SDS-PAGE analysis of BoNT/AC under reducing and non-reducing conditions. Lane 1, Broad molecular mass protein standards Myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), and ovalbumin (45 kDa, carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lane 2, BoNT/AC under non-reducing conditions; Lane 3, BoNT/AC under reducing conditions.

B. Western blot analysis of BoNT/AC under non-reducing and reducing conditions. BoNT/AC was treated with anti-BoNT/AC IgG followed by colorimetric detection as described in the methods section. Lane 1, Precision plus kaleidoscope pre-stained protein standards (250, 150, 100, 75, 50, 37, 25, 20, 15, 10 kDa); Lane 2, BoNT/AC under non-reducing conditions; lane 3, BoNT/AC under reducing conditions. Arrow points to the highly immunogenic P-250 protein.

FIGURE 3. Protection from BoNT/A intoxication by anti-BoNT/A and anti-BoNT/AC sera. **A.** Neutralizing capacity of undiluted anti-rabbit sera of BoNT/A or BoNT/AC to10 lethal doses of pure BoNT/A. **B.** Neutralizing capacity of rabbit anti-BoNT/AC and anti-BoNT/A sera to purified BoNT/A at 50% titer of BoNT/AC.

FIGURE 4. A. Genomic map of *Clostridium botulinum* strain Hall, showing the location of origin, BoNT/A cluster gene, and the gene of P-250 protein.

B. TBLASTN & GeneMark analysis of *Clostridium botulinum* strain Hall-A ATCC 3502 genome for identifying the location of the peptide sequenced from P-250 protein band. GeneMark software predicted 5 ORFs around the area that included the peptide sequence "LLDSSESITLDKTSM". The area boxed in blue color showed the longest ORF translation consisting of **359** amino acid residues.

FIGURE 5. Graph depicting the binding of BoNT/AC, Hn-33, BoNT/A, BoNT/A light and heavy chains to their respective antibodies as determined by ELISA.

FIGURE 6. Binding of BoNT/A, BoNT/AC, BoNT/A light and heavy chain to antibodies raised against BoNT/A.
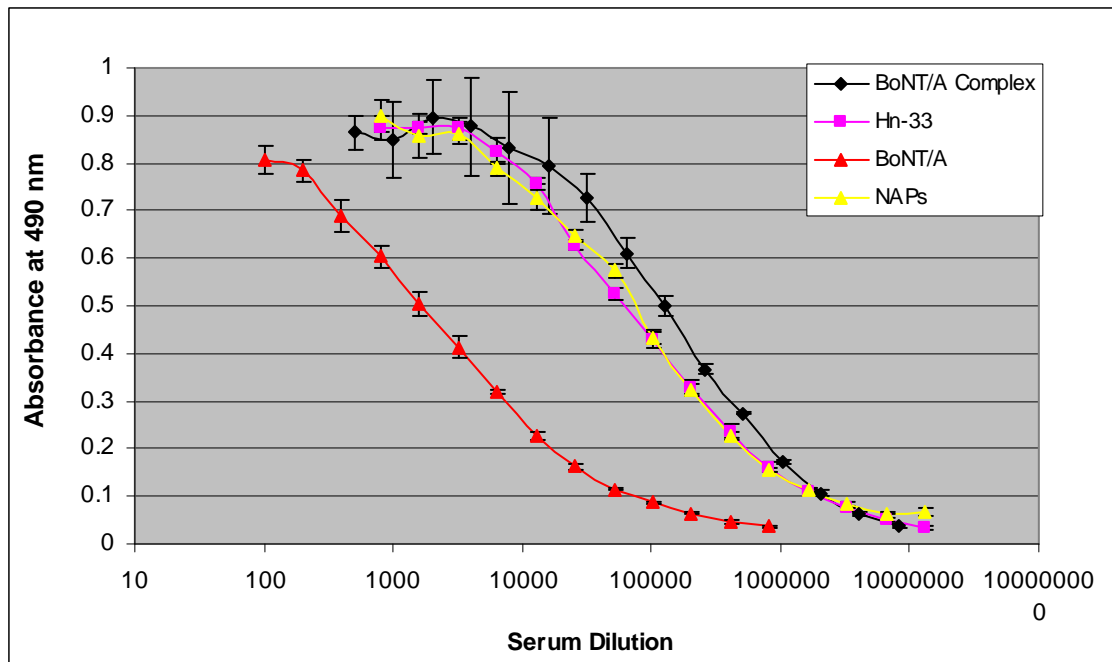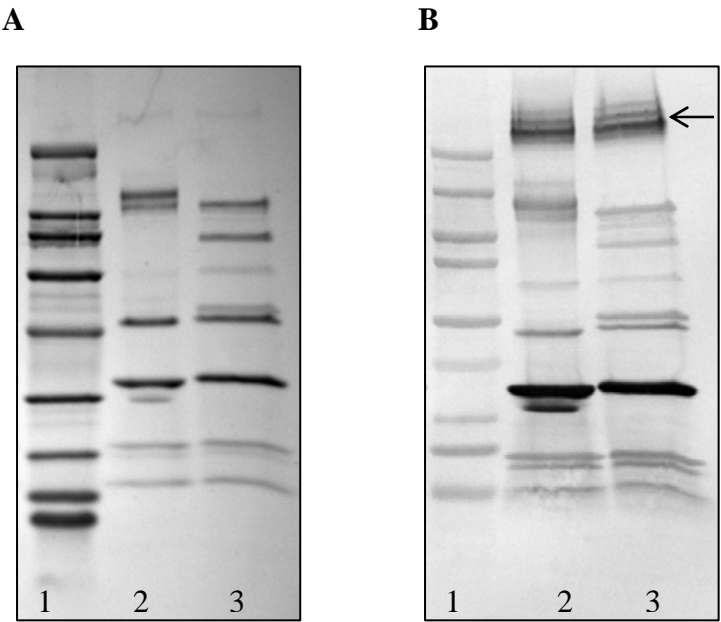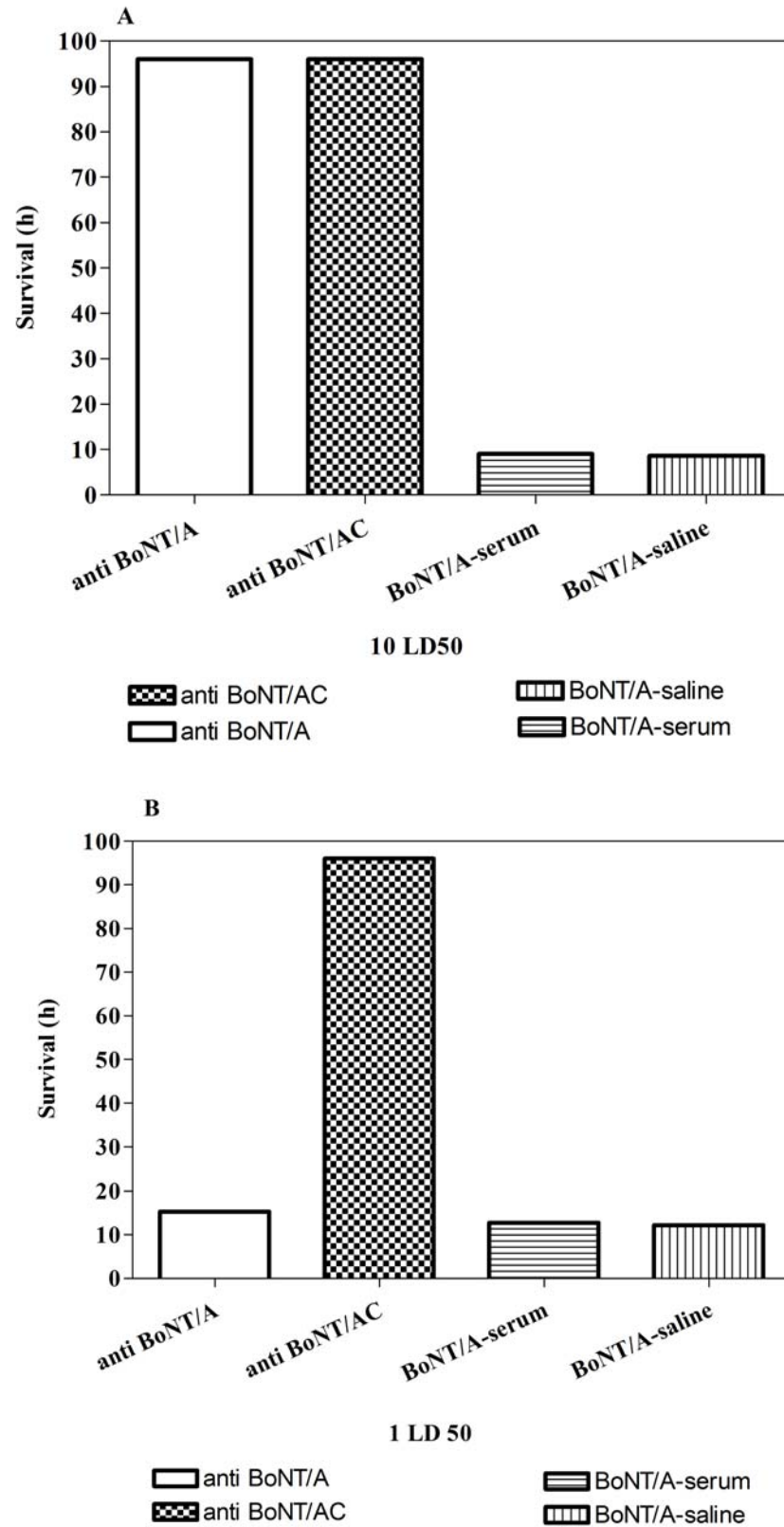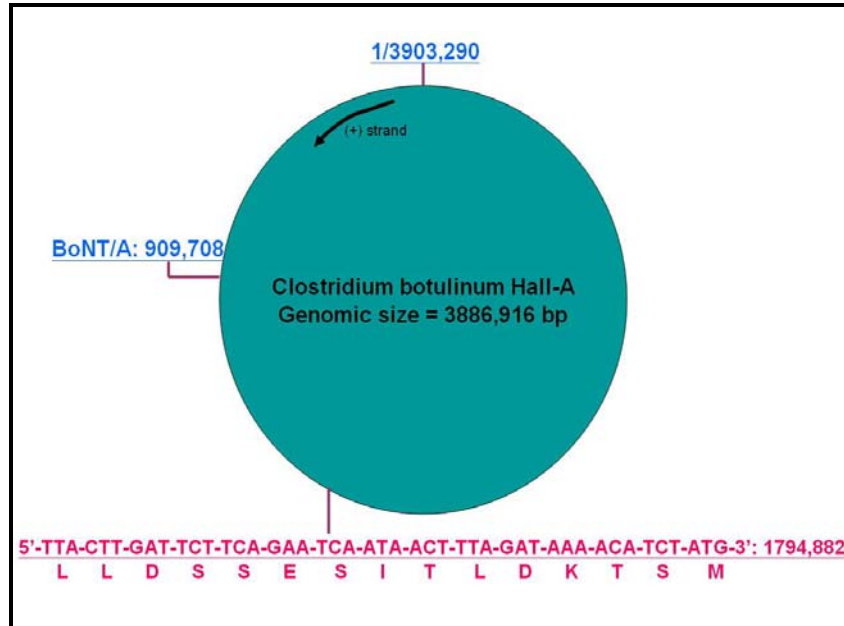
FIGURE 1

FIGURE 2

FIGURE 3

A

10 LD50

anti BoNT/AC
anti BoNT/A
BoNT/A-saline
BoNT/A-serum

B

1 LD 50

anti BoNT/A
anti BoNT/AC
BoNT/A-serum
BoNT/A-saline

18

FIGURE 4

A.

B.


```
3501   MYCT*GEF*T  CKI
3551   GYKMVSFTLS  REI
3601   KSSYNQKLRL  *RI
3651   SFLDDKRRSK  ELI
3701   YNLSML***H  GN:
3751   SKLTKIC*PL  I*1
3801   EDNTMKKKIG  LI1
3851   EKNSAKVGDM  LRI
3901   SGENVNSKFK  FTI
3951   YQRLVFEKID  LSI
4001   SITLDKTSMD  LL1
4051   VTAIKEGKAT  IT
4101   KEYDVNMQEV  EKI
4151   ASFEVREYEG  TNI
4201   LKIEKILKIQ  QPC
4251   SYCRGKGGRI  KQI
4301   IINGIYFLRG  TYI
4351   QVQLLMQLHP  AKI
```

FIGURE 5

FIGURE 6

TABLE 1. Sequence homology of P-250 amino acid sequence with different protein identified by Blast P sequence analysis.

Top 8 BLASTP results (Most Significant)	Identity / Positive Similarity	Bits Score
Bacterial Ig-like domain protein [*Clostridium botulinum* strain NCTC 2916 & Bf]	93% / 97%	309
Cell adhesion domain of [*Clostridium botulinum* strain B1 Okra]	93% / 96%	305
Hypothetical protein CLK_1112 [*Clostridium botulinum* A3 str. Loch Maree]	92% / 96%	303
Cell adhesion domain [*Clostridium botulinum* F str. Langeland]	92% / 96%	300
Hypothetical protein CLD_2020 [*Clostridium botulinum* B1 str. Okra]	85% / 91%	226
Cell adhesion domain [Bacillus sp. B14905]	59% / 72%	178
Cell adhesion domain [Lysinibacillus sphaericus C3-41]	57% / 73%	174
Ig domain protein, group 2 domain protein [*Clostridium beijerinckii* NCIMB 8052]	44% / 64%	128

Anti-apoptosis Host Reponse to the Exposure of a Component of Botulinum Neurotoxin Complex

Introduction

Clostridium botulinum type A is a gram-positive anaerobic spore forming bacterium, which secretes neurotoxin, the causative agent of the deadly neuroparalytic botulism disease (Lund, 1990; Sugii and Sakaguchi, 1977). The 150 kDa neurotoxin is secreted along with seven neurotoxin associated proteins (NAPs) which form a neurotoxin complex (Fu et al., 1998; Singh et al., 1995). Hemagglutinin is a 33 kDa protein which has been found to be a major component of the neurotoxin complex and has hemagglutination activity (Fu et al., 1998). NAPs are known to play an important role in the protection of the neurotoxin from adversarial conditions such as exposure to proteases, bile salts and acidity in the GI tract (Sakaguchi, 1983; Fujinaga *et al*., 1997; Sugii and Sakaguchi, 1977; Cai et al., 1999). However, the exact role of these NAPs is not well understood. It has been reported that NAPs play significant role in the translocation of the neurotoxin across the mucosal layer of the GI- tract (Fujinaga et al., 1997).

Previously we have shown that the Hn-33 can imitate the BoNT/A complex in hemagglutination, protection of BoNT/A against proteases, and enhancement of BoNT/A endopeptidase activity (Sharma and Singh, 1998; 2004; Cai et al., 1999). We recently showed that Hn-33 directly binding to the neuronal cells (Zhou et al., 2005). In an effort to evaluate host-response to Hn-33, we have now tested its effect on the apoptosis of neuronal cells.

Apoptosis is an active process of cell death involved in morphogenesis (Jacobson et al., 1997) and many diseases, such as cancer (Yang and Korsmeyer, 1996), and neurodegeneration (Gorman et al., 1996). The mechanism of apoptosis is highly complex. The biochemical mechanism of apoptosis can be divided into 4 stages: 1: death signals (external factors, receptor-mediated and non receptor-mediated), 2: integration or control stage (signal transduction, activation of transcription factors, induction of apoptosis related genes, release of $Ca^{2+,}$ depletion ATP), 3: the execution phase (cell dismantling, DNA degradation, expression of phagocyte, recognition molecules), 4: removal of dead cells by phagocytosis. A variety of external triggers such as oxidative stress (Kagedal et al., 2001), RNA or protein synthesis inhibitors (Martin et al., 1990), DNA damaging agents (Berrand et al., 1993). Two main integration pathways exist and converge to a common execution phase: one is death receptor pathway, another one is mitochondrial pathway.

The common methods to detect apoptosis include observation of morphological change of cells, FACS analysis of cells, analysis of DNA ladder, detection of nuclear condensation using Hochst 33342 to stain cells, and Western blot analysis of caspase-3 (Shimura et al., 1998). Dying cells that undergo the final stages of apoptosis, display signals, like phosphatidylserine on the cell surface. Phosphatidylserine is normally found on the cytosolic (inner) surface of the plasma membrane, but is redistributed during

apoptosis to the extracellular surface by a hypothetical protein known as scramblase. The AnnexinV-FITC kit uses annexin V conjugated with fluorescein isothiocyante (FITC) to label phosphatidylserine sites on the membrane surface of apoptotic cells.

Morphological observations showed the inhibitory effect of Hn-33 on the apoptosis in SH-SY5Y cells induced by 500 μM H_2O_2. In order to explore the mechanism of inhibitory effect of Hn-33 on the apoptosis in SH-SY5Y cells, further experiments need to be carried out.

Experimental Methods

Cell line and culture conditions: The human neuroblastoma cell SH-SY5Y was purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in 1:1 mixture of Eagle's Minimum Essential Medium with non-essential amino acids from ATCC (Manassas, VA) and Ham's F12 medium from Sigma (St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (ATCC, Manassas, VA) at 37°C, in a humidified 5% CO_2 incubator.

Introduction of apoptosis of SH-SY5Y cells: After seeded the cells in 25 cm^2 flask for 48 hours, the cells were rinsed with fresh serum free culture medium once, then H_2O_2 was added at final concentration of 500 μM in the serum-free medium.

Treatments of SH-SY5Y cells with Hn-33 and pepstatin A (PA, inhibitor of cathepsin D): After seeded the cells in 25 cm^2 flask for 48 hours, the cells were rinsed with fresh serum free culture medium once, and treated with 150 nM Hn-33 and 10 mg/ml of pepstatin A in fresh serum free culture medium for 2 hours. Then H_2O_2 was added at final concentration of 500 μM in the serum-free medium.

Detection of apoptosis: Apoptosis in SH-SY5Y cells was detected with Annexin V-FITC Apoptosis Detection Kit (Sigma). Annexin V-FITC kit allows fluorescent detection of annexin V bound to apoptotic cells. Apoptosis was induced in SH-SY5Y cells by 500 μM H_2O_2. The cells were washed with PBS and then washed with binding buffer (10mM HEPES, pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$). The cells were stained with annexin V-FITC (2 μg/ml) for 10 minutes at room temperature. After staining, the cells were washed with binding buffer and observed by fluorescence microscopy.

Observations

Figure 1: Apoptotic cells after exposure of cells to H_2O_2 and staining with Hochst 33342 (red staining of DNA) and Annexin V-FITC (green staining of phosphotidylserine) kit
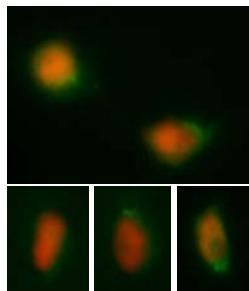
Morphology

Figuure 2. Morphological changes observed upon treatment of SH-SY5Y cells with apopstosis causing reagent, H_2O_2 in the absence and presence of Hn-33 and a well known apoptosis inhibitor, peptstatin A (PA). Results clearly show anti-apoptosis effect of Hn-33.
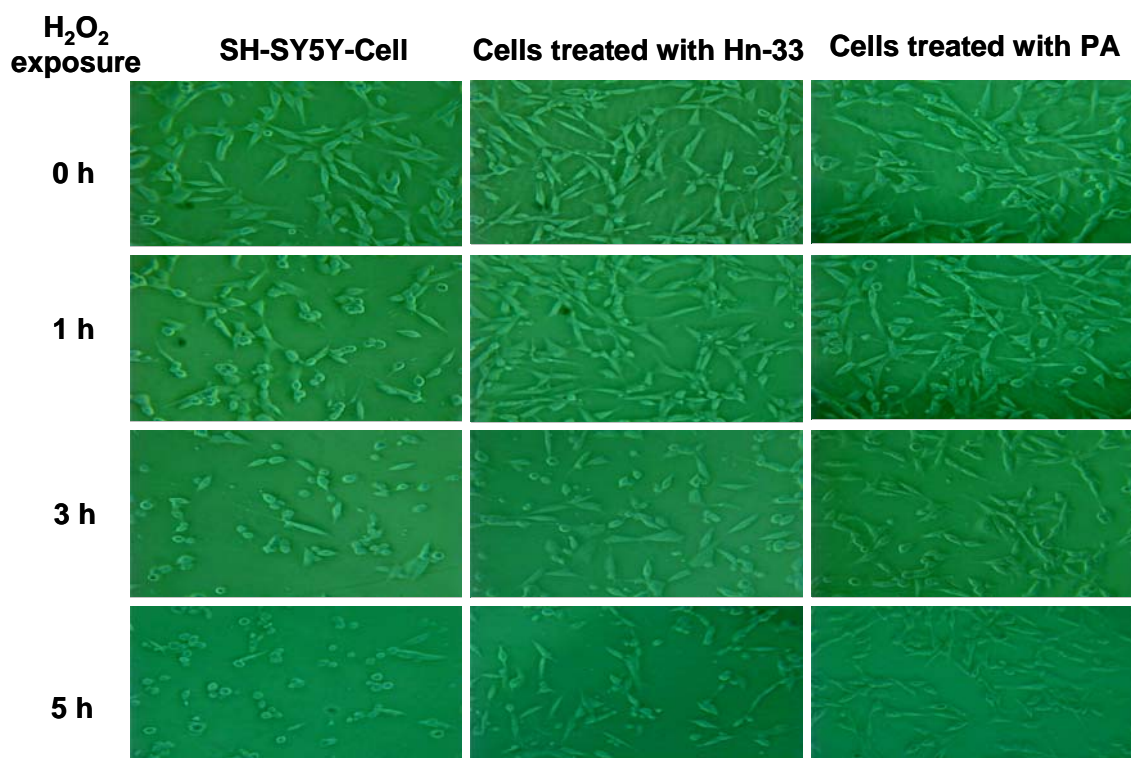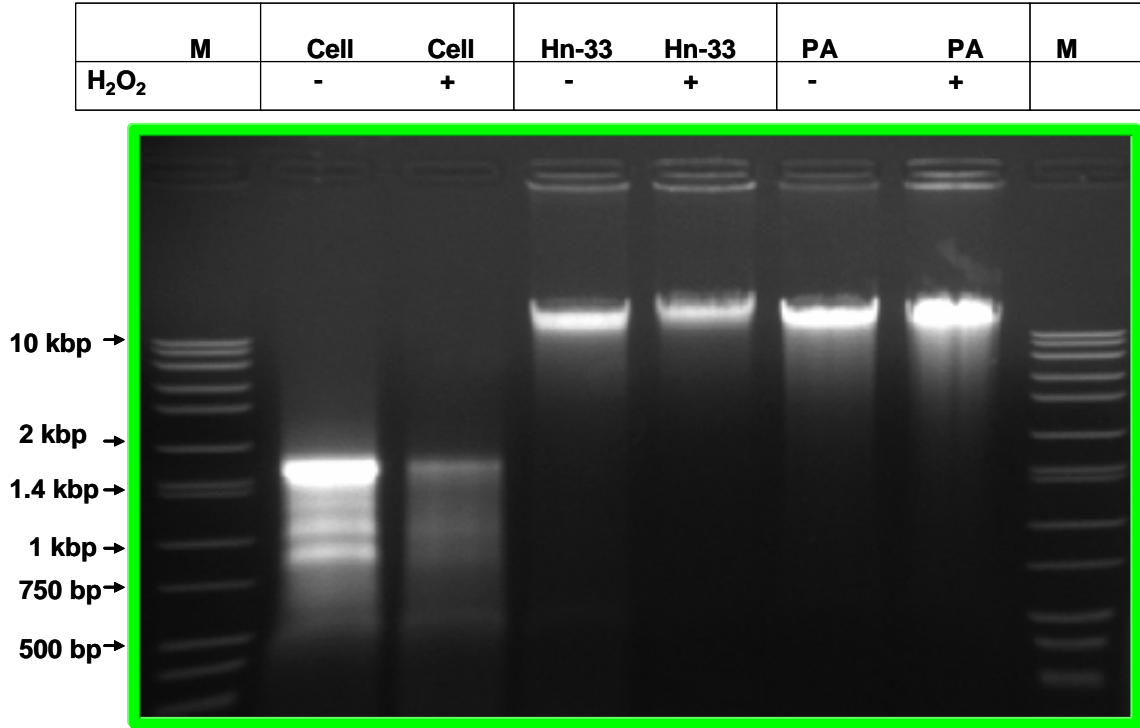
Figure 3. Analysis of DNA upon induction of apoptosis in SH-SY5Y neuronal cells in the absence and presence of Hn-33 and PA. For some unknown reasons, our control experiments also showed degraded DNA (Cell, '-' condition). We are confirming those results and conducting further experiments.

	M	Cell	Cell	Hn-33	Hn-33	PA	PA	M
H_2O_2		-	+	-	+	-	+	

Conclusions

1. Apoptosis in SH-SY5Y cells was induced by 500 μM H_2O_2 and detected with Annexin V-FITC Apoptosis Detection Kit (Figure 1).

2. Morphological observation showed that inhibitory effect of Hn-33 and pepstatin A (PA) on the apoptosis in SH-SY5Y cells induced by 500 μM H_2O_2 (Figure 2).

3. Analysis of DNA fragments is needed to be carried out again to obtain comparable results with control cells (Figure 3).

4. Inhibitory effect of Hn-33 and pepstatin A (PA) on the apoptosis in SH-SY5Y cells induced by 500 μM H_2O_2 should be identified again with Annexin V-FITC Apoptosis Detection Kit.

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Role Hemagglutinin-33 of type A *Clostridium botulinum* in the translocation of the neurotoxin across the epithelial cell and a unique response of clams to toxin exposure

1. Hn-33 enhances the translocation of BoNT across epithelial cell layer (Goal 2)

The mode of BoNT action can be divided into four steps: ingestion and intestinal absorption, binding to neuronal membranes, internalization, and intracellular biochemical action resulting into the blockage of neurotransmitter release.

The intestinal absorption of BoNT is known to be mediated by NAPs, especially Hn-33, and BoNT heavy chain (Fujinaga et al., 2004; Maksymowich and Simpson, 2004) through transcytosis. It has also been proposed that the ligand binding moiety is found in the toxin itself (Maksymowych et al., 1998; Maksymowych et al., 2004), and that there are specific receptors on the mucosal cell surface which can distinguish different BoNT types. Epidemiologically, seven serotypes of BoNT have distinct pattern of causing botulism in different organisms, including humans. One of the major reasons for this epidemiology is suggested to be the existence of different receptor(s) at the surface of intestinal mucosal cells. Furthermore, the carboxyterminal half of the heavy chain was defined as the minimum essential domain for binding and transport across human gut epithelial cells.

However, one of the reports (Maksymowych et al., 1999) clearly suggests that at low concentration of BoNT, the presence of NAPs dramatically enhances the toxicity of orally administered toxin, suggesting for a need to examine this issue carefully to develop experimental data on the kinetics of the penetration of BoNT in different forms (native and purified) relevant to physiological conditions of exposure. In addition, these results suggesting no role of NAPs in BoNT protection and penetration may be inconclusive, as the cell cultures were used for most of these experiments, and where intestinal tissue was used there was quantitative difference in the absorption of BoNT with and without NAPs (Maksymowych et al., 1999; Fujinaga et al., 2000; 2004). Recently, it was published that

only progenitor toxin, and not the purified type C botulinum neurotoxin can penetrate the cultured intestinal epithelial cells (Nishikawa et al., 2004). Thus, it is important to determine the components of BoNT complex that is necessary for binding and transport across human intestinal epithelial cells.

In this report we describe the role of Hn-33, a mjaor NAP of BoNT/A complex in the trascytosis process.
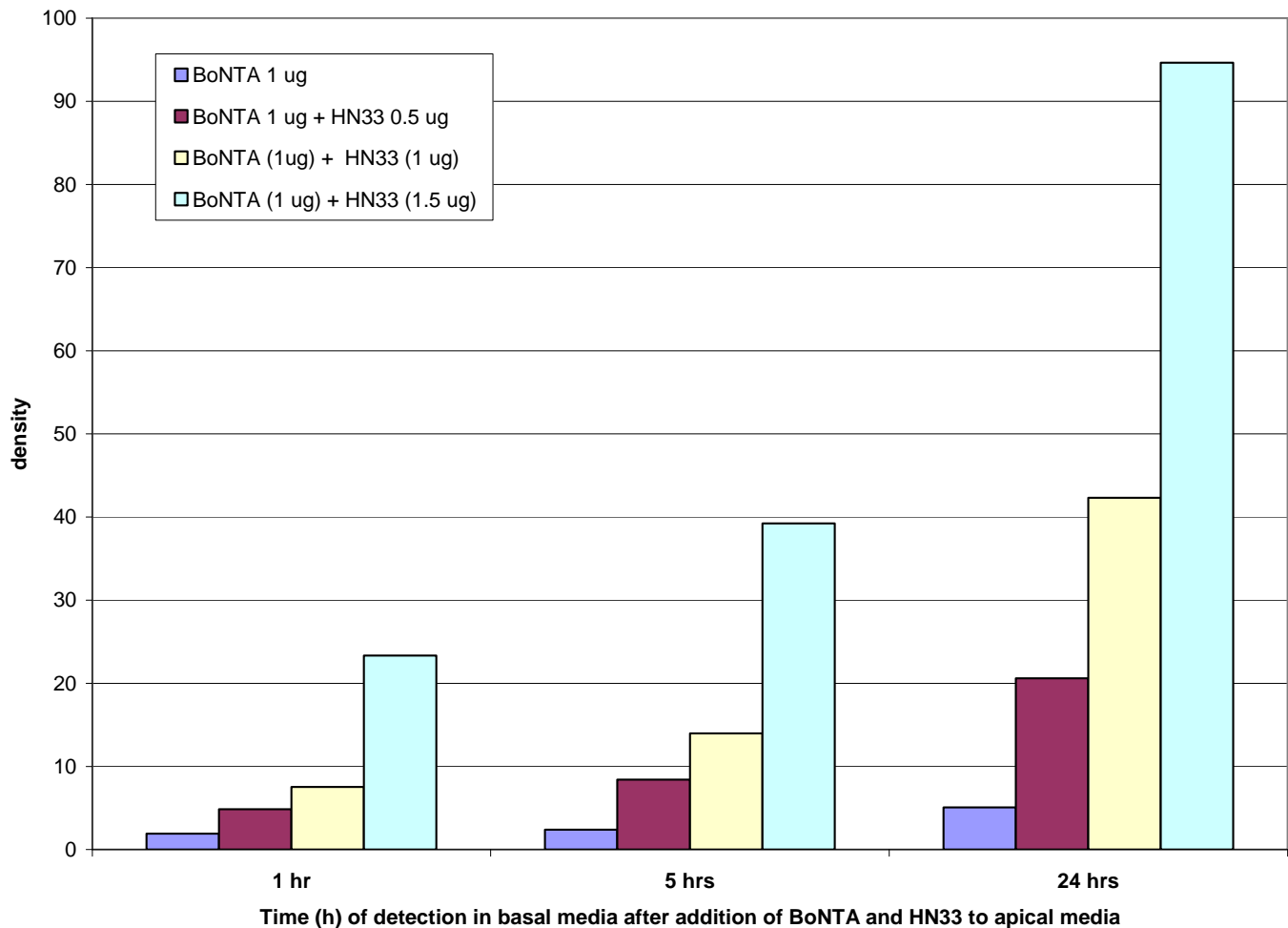
Methods

Figure 1. Translocation of BoNT/A and the effect of Hn-33 concentration on the translocation of BoNT/A across HBE cell layer.

680 nm Alexa dye was conjugated to BoNT/A (detoxified by mutating two residues in the light chain) and then 1.0 ug was added to the apical media of human bronchial epithelial (HBE) cells cultured on collagen IV-coated cell culture inserts. At time points after the addition of the protein, an aliquot was taken from the basal media and scanned on the Odyssey Infra Red Scanning Instrument. Hn-33 was labeled with the 800 nm CW fluor (LiCor, Lincoln NE), and a similar translocation experiment was performed with HBE cells (**Fig. 1**). The concentration of BoNT/A and Hn-33 was calculated from a standard Hn-33 labeled with the 680 nm or 800 nm dyes. Experiments were repeated whereby BoNT/A labeled with Alexa-680 alone, and after mixing with different concentrations of Un-labeled Hn-33, were applied to cell culture monolayers. Translocation of BoNT/A-680 was monitored.

Results and Conclusions

The translocation data was collected over a period of 24 hours, and results show that translocation of BoNT/A in the presence of Hn-33 continues to increase with

concentration (**Fig. 1**). Molar ratios of BoNT/A to Hn-33 used were 1:2.3, 1:4.5, and 1:6.75. Based on the previous experiment (Report 4 dated May 18, 2007) BoNT/A and Hn-33 seem to translocate at a similar rate, the mixture of the two enhances the translocation of BoNT/A and Hn-33 each by about three-fold. The effect of Hn-33 at 1:6.75 molar ratio seems to be significantly higher than predicted from the linear increase, which appears to be the case for the first two concentrations. This observation indicates that that the Hn-33 at higher concentration is not only binding and enhancing the ability of the toxin to transcytose more effectively, rather it perhaps translocates itself carrying the BoNT/A. As we wrote in our earlier report we for the first time have demonstrated a role of a NAP directly in the translocation of BoNT in a kinetic and concentration dependent manner. This is a function in addition to the protection and enhancement of endopeptidase activity of BoNT by Hn-33 (Sharma and Singh, 1998; 2004). Earlier work from Lance Simpson's group showed that BoNT by itself was capable of translocating, which we confirm, but now show that Hn-33 and can enhance that translocation on both short-term and long time scale basis.

2. Visualization of internalization of Botulinum toxin and Hn-33 in HT-29 cells

In order to collect direct evidence of the entry of both BoNT/A and particularly of the Hn-33, we labeled these proteins with fluorescent probes, and examined their entry and movement in the HT-29 cells.

The human colon carcinoma epithelial cell line HT-29 was purchased from the American Type Culture Collection. The cells were grown in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in 5% CO_2 atmosphere.

For Confocal laser scanning microscopy analysis, HT-29 cells were grown on culture slide flasks from Nalge Nunc International. The cells were washed with fresh serum free McCoy's 5A medium and incubated with either FITC-labeled detoxified recombinant BoNT type A (DRBoNT/A) or with FITC-labeled recombinant Hn-33 (100 nM) in serum free McCoy's 5A medium at 4 °C for 90 min to enhance cell surface binding and prevent receptor mediated endocytosis. The cells were then incubated at 37 ºC for 30 min to trigger internalization of the labeled proteins in the cells. The cells were washed with PBS twice and fixed with 3.7% paraformaldehyde for 15 min. The paraformaldehyde was decanted off, followed by washing twice with PBS only. Cover glasses were mounted on slides and the cells were examined using Leica instrument model TCS SP5.

Results:
A. Internalization of FITC-labeled DRBoNT and Hn-33 into HT-29 cells after binding at 37 ºC.:

As shown **Figs. 2 and 3**, within 30 min of initializing internalization at 37 ºC, DRBoNT and Hn-33 were clearly observed inside the cell. However, DRBoNT was internalized to a lesser degree compared to Hn-33, suggesting that the NAPs are essential for effective adsorption of the toxin to the cell surface followed by internalization.
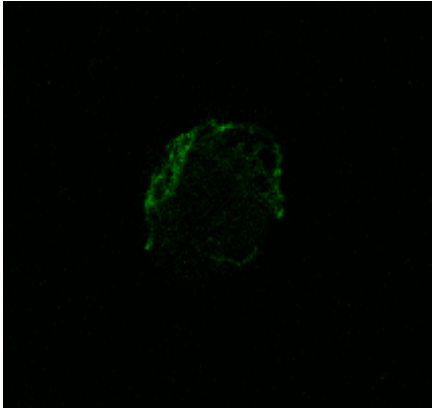
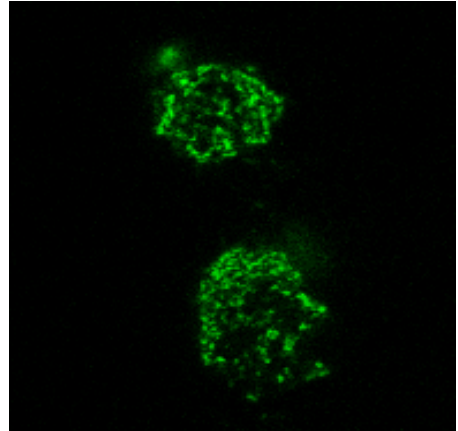Figure 2. Internalization of FITC-labeled DRBoNT into HT-29 cells

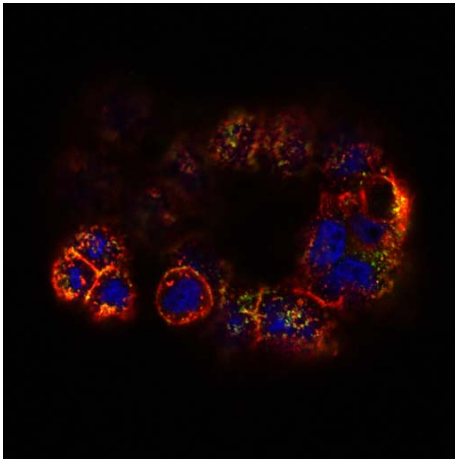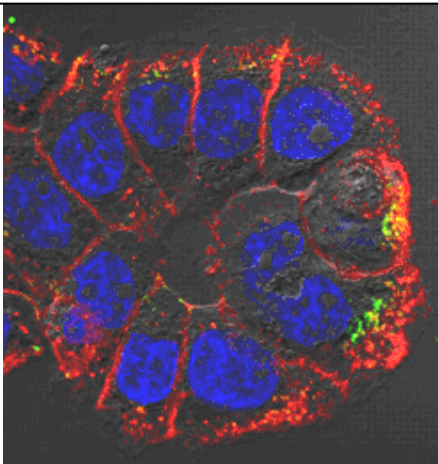Figure 3. Internalization of FITC-labeled Hn-33 into HT-29 cells

The experiments were also carried out by treating HT-29 cells with FITC-labeled DRBoNT/A or with FITC-labeled recombinant Hn-33 (100 nM) as described above followed by staining the cells with membrane tracking dye and nuclear staining dye from Sigma-Aldrich Co (**Figs. 4 and 5**). These dyes were added to the cells 30 min prior to fixing the cells. The cells were fixed as outlined above and examined using Carl-Ziess confocal microscope. Results show that both DRBoNT/A and Hn-33 enter the cytoplasm, but not the nucleus of HT-29 cells.

Figure 4. Internalization of FITC-labeled DR BoNT into HT-29 cells

In another set of experiments, HT-29 cells that were grown on culture slide flasks (Nalge Nunc International) and washed with fresh serum free McCoy's 5A medium, were incubated with FITC-labeled recombinant Hn-33 (100 nM) in serum free McCoy's 5A medium directly at 37 °C for varying time periods (2 h, 5 h, 24h) to trigger internalization of the labeled proteins into the cells. The cells were washed with PBS twice and fixed with 3.7% paraformaldehyde for 15 min. The paraformaldehyde was decanted off, followed by washing twice with PBS only. Cover glasses were mounted on slides and the cells were examined using Carl-Ziess Confocal microscope.

Figure 5. Internalization of FITC-labeled Hn-33 into HT-29 cells

B. Internalization of FITC-labeled Hn-33 into and transcytosis across HT-29 cells:

As shown in **Figure 6 and 7**, within 2 h of initializing internalization at 37 ºC, Hn-33 had entered the cells. After 24 h incubation at 37 °C, FITC-labeled Hn-33 was localized on the outside of the cell surface which may indicate its release from the cell (**Fig. 8**)
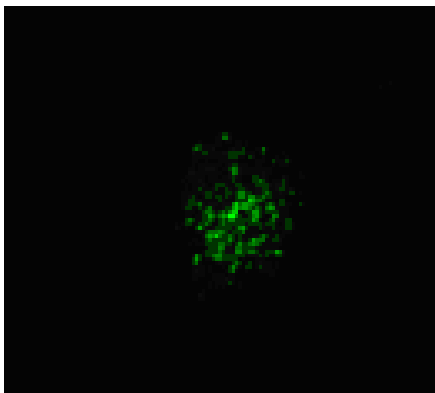

Figure 6. Internalization of FITC-labeled-Hn-33 into HT-29 cells after 2 h incubation at 37 °C
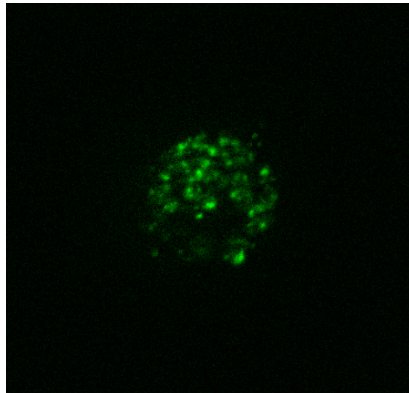

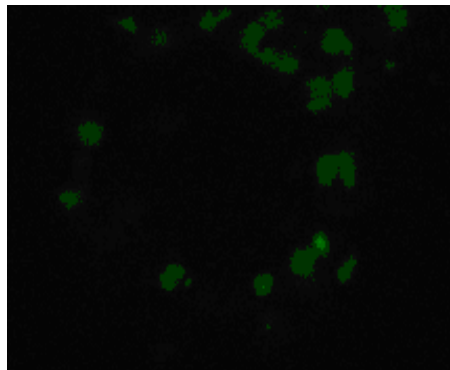Figure 7. Internalization of FITC-labeled-Hn-33 into HT-29 cells after 5 h incubation at 37 °C

Figure 8. Internalization of FITC-labeled-Hn-33 in HT29 cells after 24h incubation at 37 °C

3. **Unique Physiological Response of Northern Quahog to Botulinum Neurotoxin**

Previsouly, we had observed that when high doses (more than thousand mouse LD_{50} units) of type A botulinum neurotoxin (BoNT/A) were injected in the anterior adductor muscles of northern quahog, *Mercenaria mercenaria*, to test its effect on muscle paralysis and lethality to quahogs, no muscle paralysis or mortality was observed, and the quahogs responded to the toxin injection with ejection of mucus and browning of tissues. In order to understand the molecular basis of quahog's resistance to BoNT, we have examined binding and internalization of BoNT/A into nerve cells of quahog using immunofluorescence staining and confical microscopy. We labeled BoNT/A with FITC (fluorescein-5-isothiocyanate) and found that labeled FITC-BoNT/A binds and enters into the quahog nerve cells. Thus the physiological and perhaps biochemical response in quahog is likely to result from specific binding of the toxin to the nerve cells. These findings may be relevant to developing an invertebrate animal assay model for assay and diagnostics of the botulinum biothreat.

Injection of BoNT into quahogs

Quahogs (clams) were purchased from Captain Frank's Seafood, New Bedford, MA. (Length 80 ~ 85 mm; Height 75 ~ 80 mm; Weight 145 ~ 160 g). They were maintained in laboratory for 2 weeks in seawater and aeration was provided by air bubbling using Spectra/Chrom MP- 1 Pump; room temperature varied from 24 ^{o}C ~ 27 ^{o}C . Clams were not fed, and subjected to natural light cycle.
The clams were divided into two groups. The controls received 200 µl of buffer (20 mM sodium phosphate buffer, pH 7.9) injection into the anterior adductor muscle by inserting a hypodermic needle through a notch filed into the edge of the shell. The experimental group received 20 µg and 96 µg of BoNT/A complex (in 200 µl of 20 mM phosphate buffer, pH 7.9) injection into the anterior adductor muscle using a 1 ml Sub Q (0.45 mm X 16 mm) syringe (BD & CO., Franklin Lakes, NJ).

Isolation and preparation of quahog nerve cells and immunofluorescence staining

The soft tissues of quahogs were removed from its shells and were dissected out quickly on ice to collect the nervous tissue (cerebral, pedal and visceral ganglions and the connecting nerves). The collected nervous tissues were washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.3).
BoNT/A was labeled with FITC (fluorescein-5-isothiocyanate) using FluoroTag FITC Conjugation Kit from Sigma-Aldrich (St. Louis, MO) (Zhou et al., 2005), using the instruction manual provided by the supplier.

The quahog nerve tissues were placed on glass slides and smeared and smashed with a coverslip in order to observe quahog nerve cells under a micrpscope, and then fixed on glass slides for 20 min with 4% paraformaldehyde (PFA) in PBS and permeablilised with 0.2% Triton X-100 in PBS for 15 min. The cells on the slides were subjected to immunofluorescence staining as described in our previously published paper (Zhou et al., 2005). This experiment was performed at room temperature (24 ~ 27°C). The slides were

washed with PBS and incubated with 1% (w/v) bovine serum albumin (BSA) in PBS for 1 hour, then followed by incubation with 5.3 µM FITC-labeled BoNT/A for 30 min, respectively. After washing the slides with PBST (0.05% Tween 20 in PBS) for five times, cover-slips were mounted on the slides with a drop of Fluoromount-G (Southern Biotechnology Associates, Inc., Birmingham, AL), according to the manufacturer's instructions. Fluorescence images were acquired with a Nikon Eclipse E600 MVI microscope equipped with a digital camera controlled by the software "SPOT" (Diagnostic Instruments. Inc., Sterling Heights, MI), or confocal microscope (Zeiss 410 confocal laser scanning Leica TCS SP2 AOBS spectral confocal microscope, renaissance software from Microcosm, Columbia, MD).

Results

Toxicity Test

Quahogs were injected with different doses of botulinum neurotoxin- BoNT/A complex, BoNT/B complex and BoNT/F complex for varying time intervals and no mortality was observed even after injecting very high doses (180 µg of BoNT/A complex and 150 µg of BoNT/F). Moreover, there was no visible dysfunction observed in the quahog's ability to shut its shell, indicating no signs of muscle paralysis.

Morphology

The quahogs administered with 20 µg and 96 µg of BoNT/A complex for 3, 6 and 9 days exhibited browning of the body color and an apparent morbidity in the flesh (**Figure 9**). These changes were more pronounced in the quahogs injected with 96 µg of BoNT/A complex. On the other hand, the controls receiving vehicle (the phosphate buffer) showed cream colored body with more contractile activity in the adductor muscles and foot as compared to BoNT injected quahogs.

Turbidity Test

The first reaction of the BoNT/A complex injected quahogs was that they started ejecting out some milky mucus (within 2-3 hours after injection) from the small pore formed towards anterior adductor muscles. Due to this activity, the color of the seawater becomes milky within the first day after receiving the BoNT injection as compared to controls. The water from containers was checked for absorbance at 600 nm on the spectrophotometer, it exhibits a significant increase in the turbidity in experimental animals (**Figure 10**).

Binding of labeled BoNT/A with Quahog Nerve Cells

The quahog nerve cells incubated with FITC-labeled BoNT/A showed fluorescence signals (**Figure 11**). In another experiment, quahog nerve tissues that had remained intact after smashing with coverslips were directly blocked with 1% BSA without fixing them with 4% paraformaldehyde (PFA) and in PBS and permeabilized with 0.2% Triton X-100

in PBS also showed fluorescence signals after incubated with FITC-labeled BoNT/A (**Figure 12**).

Binding and internalization of BoNT/A into fixed nerve cells of quahog were observed under fluorescence and confocal microscope. Binding of BoNT/A to live quahog nerve tissue and cells were observed under confocal microscope.

Our experimental findings clearly indicate that the quahogs do not die even at very high dose (180 µg, equivalent to a dose of 18 million mouse LD_{50}) of BoNT/A, but they undergo some distress showing ejection of mucus as an immediate response and browning of the body color. Decrease in GST activity after receiving 20 µg and 96 µg of BoNT/A is biochemical response either to the neurotoxin or to the distress caused by the neurotoxin.

Browning of the body color in *Mercenaria mercenaria* has been reported as an immediate response of environmental contamination, and a role of red glands is suggested in detoxification by Zaroogian et al. (1989). The responses of quahogs towards BoNT/A may be the result of the general detoxification due to the extreme potency of the toxin (Lamanna 1959; Singh 2000).

Our observation that the toxin clearly binds and enters the quahog nerve cells suggests that either there is mechanim in quahogs to inhibit the BoNT/A endopeptidase activity, or the substrate for the enzyme is not compatible for cleavage by the endopeptidase. Answers to these questions would important milestones in not only understanding the mechamism but also the basis of physiological host response in quahogs.

Effect of botulinum neurotoxin on quahog could provide a convenient biological assay system, based on the mucus release and tissue browning. However, further investigations are required in order to fully understand how the BoNT acts on the tissue to trigger physiological response of quahogs.
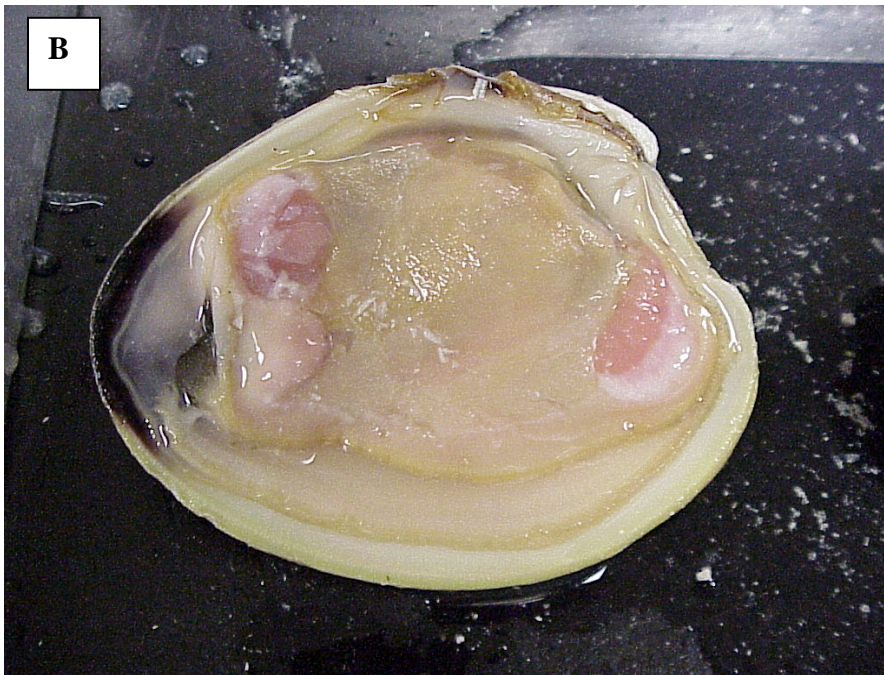
Figure 9. Inner morphology of quahog after injection with BoNT/A complex. **(A)** Quahog injected with 96 µg of BoNT/A complex after 6 days. **(B)** Quahog control injected with vehicle (20 mM phosphate buffer, pH 7.9).
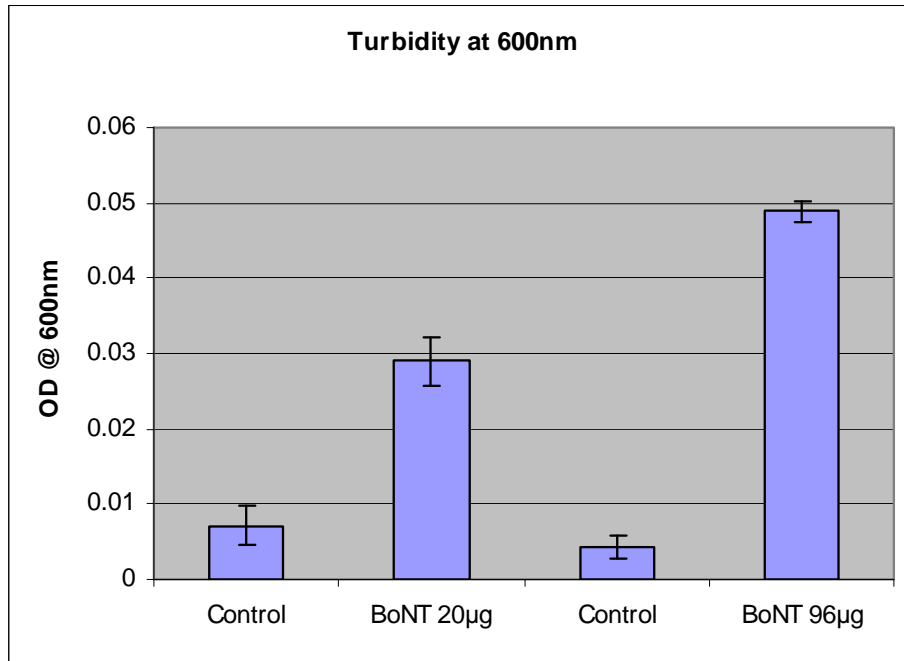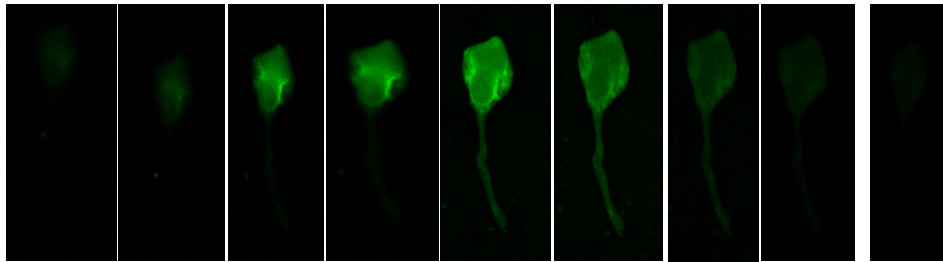
Figure 10. Turbidity of seawater in the surrounding of quahog injected with 96 g BoNT/A complex. In control experiments, the quahogs were injected with 20 mM phosphate buffer, pH 7.9.

Quahog nerve cell

FITC-BoNT/A
Confocal microscope
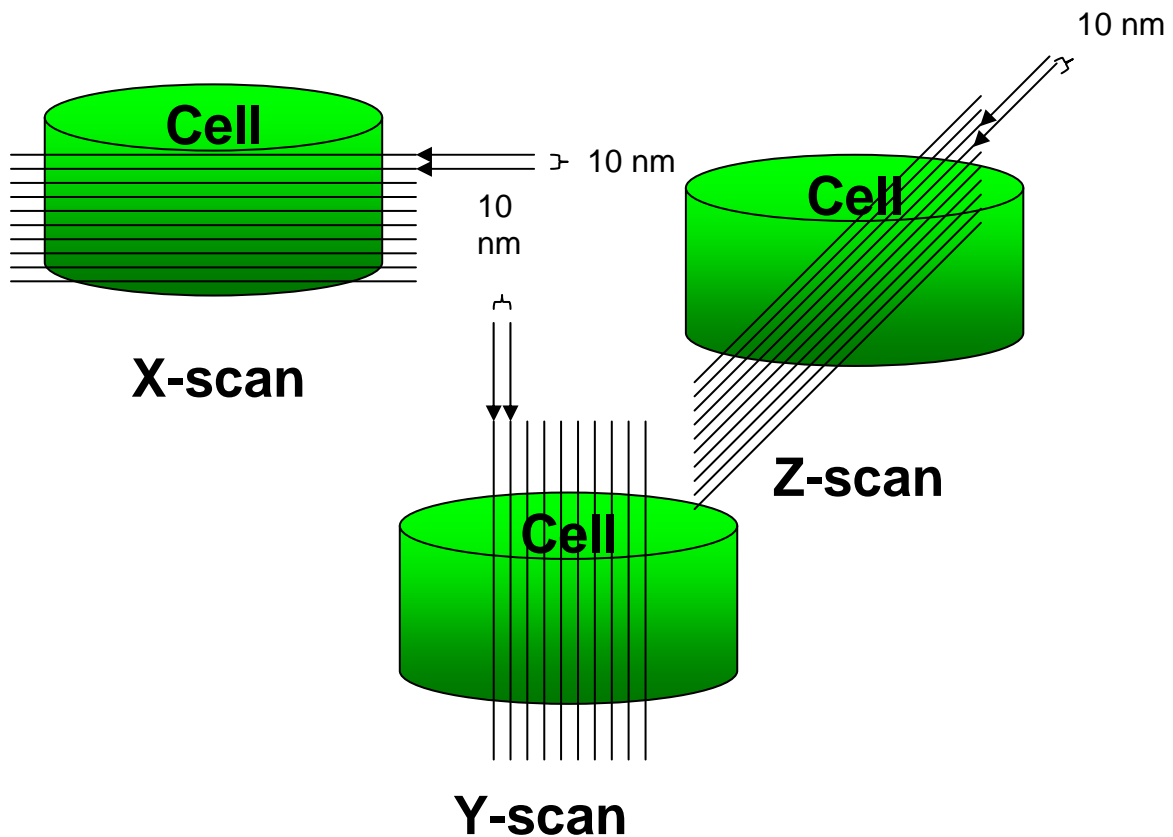
X-scan

Z-scan

Y-scan

Figure 11. Immunofluorescence detection of FITC-labeled BoNT/A binding to quahog nerve cells using confocal microscope. The quahog nerve cells were fixed and permeabilized as described in the Experimental Procedures. The quahog nerve cells were incubated with 5.3 µM FITC-labeled BoNT/A for 30 min after blocking with 1% BSA.
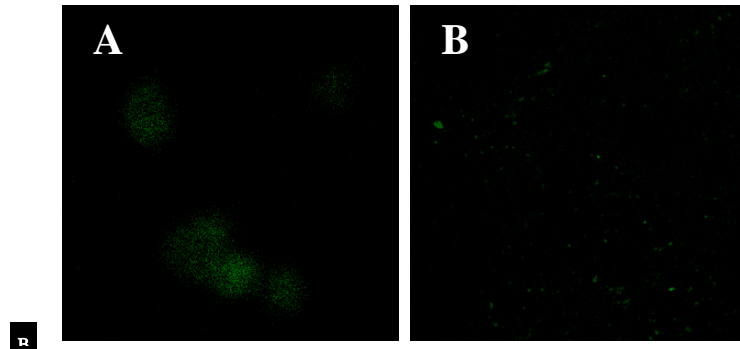
Quahog nerve tissue

Figure 12. Immunofluorescence detection of FITC-labeled BoNT/A binding to quahog nerve tissues using confocal microscope. The quahog nerve tissues were directly blocked with 1% BSA without being fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS as described in the Experimental Procedures. The quahog nerve tissues were incubated with 5.3 µM FITC-labeled BoNT/A for 30 min after blocking with 1% BSA (A), incubated with FITC-conjugated IgG (B).

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Hemagglutinin-33 as a Potential Heat Shock Protein in Clostridium botulinum

INTRODUCTION

Growing bacterial cells encounter with a series of environmental changes under natural ecosystem, e.g., high temperature, pH and nutritional conditions (Neidhardt *et al*., 1984). Several of these stressful conditions trigger enhanced synthesis of a set of highly conserved proteins called heat shock proteins (HSPs) (Ang *et al*., 1991; Lindquist and Craig, 1988; Morimoto and Santoro, 1998; Neidhardt *et al*., 1984 and Pich**, *et al*.**, 1990). Some of the HSPs are constitutively expressed in the cell at threshold level, and their synthesis is enhanced at high temperature (Hartl *et al*., 1994).

Increased synthesis of HSPs has been correlated with an increased tolerance against thermal and other forms of stressors (Ang *et al*., 1991; Li and Werb 1982). HSPs are also involved in assembly and disassembly of protein complexes thus acting as molecular chaperones. In several pathogenic bacteria, HSPs play a protective role and influence the pathogenicity of the organism (Macario, 1995; Sokolovic *et al*., 1990). Identification of such virulence-associated proteins is critical to the understanding of the host-pathogen relationship (Johnson *et al*., 1991). Moreover, detailed knowledge of the HSPs pertaining to their role in diverse patho-physiological functions could provide clues to their possible use as vaccine carriers (Johnson *et al*., 1991).

Clostridium botulinum type A is a gram-positive anaerobic spore forming bacterium, which secretes neurotoxin, the causative agent of the deadly neuroparalytic botulism disease (Lund, 1990; Sugii and Sakaguchi, 1977). The 150 kDa neurotoxin is secreted along with seven neurotoxin associated proteins (NAPs) which form a neurotoxin complex (Fu *et al*., 1998; Singh *et al*., 1995). Hemagglutinin is a 33 kDa protein which has been found to be a major component of the neurotoxin complex and has hemagglutination activity (Fu *et al*., 1998). NAPs are known to play an important role in the protection of the neurotoxin from adversarial conditions such as exposure to proteases, bile salts and acidity in the GI tract (Sakaguchi, 1983; Fujinaga *et al*., 1997; Sugii and Sakaguchi, 1977; Cai *et al*., 1999). However, the exact role of these NAPs is not well understood. It has been reported that NAPs play significant role in the translocation of the neurotoxin across the mucosal layer of the GI- tract (Fujinaga *et al*., 1997).

In the present study, we report that the 33 kDa protein of *Clostridium botulinum* is a heat shock cognate protein. Using antibodies against neurotoxin complex, it has been shown that the 33 kDa protein is a major hemagglutinin component of neurotoxin complex whose synthesis is considerably increased at elevated temperature, as compared to the control growth temperature conditions.

MATERIAL AND METHODS

Organism and growth conditions.

Stock culture of *Clostridium botulinum* was prepared from a single colony, and was further sub cultured after proper identification. *Clostridium botulinum* type A cells were grown anaerobically in N-Z amine-based growth medium according to the procedure described previously by Fu et al. (1998).

Heat shock conditions and analysis of protein profile.

Culture for heat shock experiments were grown to mid log phase (OD 0.4-0.6 at 580 nm) at 37 °C in an incubator and a part of the culture was shifted immediately to 45°C in a prewarmed glass vessel for stipulated time period. Following shifting the cells at 37 and 45 °C, 100 ml sample was withdrawn at 0, 30, 60, 120 and 180 min from cultures at each temperature, and cellular extract was prepared according to Shukla and Singh (1999).

Preparation of whole cell extract and Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis.

The cells were harvested by centrifugation (8000 x g for 15 min at 4°C). Pellet was washed with 0.5 ml sterile distilled water and was resuspended in 300 μl extraction buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β- mercaptoethanol, pH 8.0). The cells were lysed by sonication using 5 mm probe for 3 min in 30 sec on/off cycle. The crude cellular extract was centrifuged at 10,000 x g for 10 min. The supernatant was withdrawn carefully and was used for further experiments.

SDS-PAGE was performed on a gel composed of 12.5 % homogenous gel (Mini-Protean II, Bio-Rad, Hercules, CA). The protein samples were prepared by mixing one volume of sample and 4 volumes of reducing sample buffer. Samples were boiled for 4 min and 30 μl sample (250 μg total protein) was loaded per gel slot. The gel was run at 180 volts for 45 min at room temperature and was stained with 0.1% coomassie blue.

Preparation of neurotoxin complex and immunoblot analysis.

Neurotoxin complex was prepared as described by Fu et al. (1998). Cells were grown at 37 ° and 45 °C for 12 hr and were acidified with 3 N H_2SO_4 and were allowed to precipitate. After 2 hr the precipitate was centrifuged at 2400 x g for 20 min at 4 °C. Supernatant was discarded and pellet was used as the neurotoxin complex extraction after redissolving it in 0.05 M citrate buffer, pH 5.5.

Protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA). For immunoblot analysis, neurotoxin complex was mixed with four volumes of reducing sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.2 M β-mercaptoethanol, 0.02% bromophenol blue), and boiled for 4 min. 30μl sample (250μg total protein) was loaded per gel slot on a 12.5 % homogenous gel. The electrophoretically resolved proteins were electroblotted on to 0.45 μm nitrocellulose membrane for 1 h at 100 volts using a Bio-Rad Mini Gel Blotter (Bio-Rad, Hercules, CA).

After electroblotting, the membrane was washed 3 times with 1X PBS buffer, (Phosphate Buffered Saline; 130 mM NaCl, 2.8 mM KCl, 5 mM Na_2HPO_4, 5 mM KH_2PO_4), pH 7.3, containing 0.1 % Tween 20. Subsequently, the membrane was blocked with 3 % BSA in 1X PBS buffer, pH 7.3, for 1 h. After blocking, the membrane was washed 5 times with PBS buffer and incubated overnight with primary antibodies (horse IgG developed against neurotoxin complex type A) at 1: 1000 dilution at 4°C overnight. The membrane was again washed 5 times with 1X PBS buffer, pH 7.3 and incubated with peroxidase conjugated secondary antibodies (rabbit- antihorse IgG) at 1: 1500 dilution for 1 h at room temperature (25°C). Finally the membrane was washed 5 times with 1 X PBS buffer, pH 7.3 and immuno detection was carried out as described by Fu et al. (1998).

RESULTS

Effect of elevated temperature on the growth of *Clostridium botulinum* type A.

Upon temperature shift- up from 37 to 45°C the growth of the cells was considerably reduced after 4 hours and the growth pattern was totally altered as compared to the control (37 °C) (**Fig.1A**). However, shifting of the cells from 37 to 45 °C for 60 min resulted in an alteration of the growth pattern at 45 °C (**Fig. 1B**). At 37 °C the exponential phase of the cells started after 4 hours while at 45 °C the cells entered into exponential phase after 6 hours of lag period (**Fig.1A**).

Impact of elevated temperature on induction and synthesis of 33 kDa protein.

To examine the induction and synthesis of 33 kDa protein at elevated temperature, one part of the mid log phase culture of *Clostridium botulinum* type A was kept at 37 °C (control), and another part was shifted to 45 °C (heat shock) for 0, 30, 60 and 180 min. The pattern of protein synthesis was analyzed on a 12.5% homogenous SDS-Polyacrylamide gel electrophoresis. Equal amount of proteins was loaded per lane to compare the results precisely. As shown in **Fig. 2**, the synthesis of 33 kDa polypeptide was enhanced 2-fold at 45 °C as compared to 37 °C (**Fig. 2**, lanes 2-4), after 120 min of heat shock period (**Fig. 2**, lanes 6-9). The induction of 33 kDa protein at normal and elevated temperature was further compared by isolating the neurotoxin complex from the cells grown at normal and elevated temperature. The data showed that the synthesis of Hn-33 was significantly enhanced at elevated temperature as compared to control. Furthermore, synthesis of 9 other proteins with apparent molecular weights of 116, 80, 72, 70, 62, 53, 40, 27 and 14 kDa were induced at the elevated temperature (**Fig. 2**, lanes 6-9). The synthesis of all these stress proteins was enhanced throughout the stress period.

Identification of 33 kDa protein as hemagglutinin component of the neurotoxin complex.

To examine if 33 and 53 kDa proteins, whose synthesis at 45 °C, was significantly induced are components of type A, *C. botulinum* neurotoxin complex (**7, 3**), an immunoblot analysis was carried out with antibodies against the whole neurotoxin complex. On the SDS-PAGE gel with Coomassie blue staining, two major protein bands at 33 and 48 kDa and minor protein bands at 14 and 18 kDa, were observed for cells grown at 37 °C (Fig. 3A, lane 2). Soluble proteins extracted from cells grown at 45 °C, showed major bands at 14, 33, 48, 53 and 80 kDa, and minor bands at 17 kDa (Fig 3A, lane 3). The level of 33 kDa protein had increased by 75 % when cells were moved from 37 °C to 45 °C (**Fig. 3A**). Immunoblot blot analysis of these gels with horse anti-

neurotoxin complex IgG indicated a strong immuno-reactivity with 33 kDa, and a weak immuno-reactivity with the 53 kDa proteins (Fig. 3B, lane 3) in cells grown at 45 °C as compared to cells grown at 37 °C (**Fig. 3B**, lane 2). The antibodies also reacted, although very weakly, with the 48 kDa proteins of cells grown both at 37 °C and 45 °C (Fig. 3B, lanes 2 and 3). The reaction with the 48 kDa protein may be due to non-specific binding, particularly due to the abundant nature of this protein (Fig. 3A). These results suggest that 33 kDa protein of the neurotoxin complex may be the heat shock proteins.

DISCUSSION

The data presented in this paper indicate that *Clostridium botulinum* type A responds to high temperature by altering its growth rate and protein synthesis, and adapts to a new growth pattern appropriate to the prevailing environment, similar to other organisms (Allen *et al*., 1988; Ang *et al*., 1991). The synthesis of at least 10 proteins with apparent molecular weights of 116, 80, 72, 70, 62, 53, 40, 27 and 14 kDa was enhanced, and thus these proteins fall in the category of HSPs. The synthesis of 33 kDa protein was enhanced by 2- fold under heat shock conditions with reference to the control. It is notable that the 33 kDa protein is also produced under normal growth conditions at basal level and is induced when the cells are shifted to elevated temperature (Figs. 2 and 3). Thus it seems that the 33 kDa protein may have some physiological role in the cell compartment under normal as well as stressful conditions, such as repair and protection of other cellular proteins (Shukla et al., 1997; Vickery *et al*., 1997).

The 33 kDa protein is one of the main components of the neurotoxin complex, which also exhibits hemagglutinin activity and plays a key role in protecting neurotoxin from acidic pH of stomach, bile salts, and proteases (Shukla and Singh, 1999; Fu et al., 1998; Sakaguchi, 1983; Sharma and Singh, 1998). The Hn-33 is the only protein in the neurotoxin complex that was found to be considerably induced by the heat shock treatment. The 53 kDa protein was also enhanced but to a lesser extent (Fig. 3). This was confirmed by immunoblotting of the protein induced at 45 °C with antibodies raised against the type A botulinum neurotoxin complex (Fig. 3). Because Hn-33 is produced constitutively as well as under temperature stress conditions, we have classified it as a heat shock cognate protein (Vickery *et al*., 1997). The physiological role of Hn-33 in the toxico-infection of botulism is already established. It is known to protect the neurotoxin against adverse environmental conditions such as proteases of the GI-tract (Sharma *et al*., 1999; Sharma and Singh, 1998). In addition, Hn-33 has been demonstrated to assist in the translocation of the neurotoxin across the mucosal layer of the intestine (Fujinaga *et al*., 1997). It also directly binds and protects the neurotoxin against acidic pH and proteases of the G-I tract (Veinger *et al*., 1998; Sugii and Sakaguchi, 1977).

A notable observation made during this study was the presence of at least four NAPs 53, 33, 17 and 14 kDa at early stages of the bacterial growth (12hr), when the neurotoxin itself was not present in a significant amount (Fig. 3A). Neurotoxin has been reported to appear after 15 hr of bacterial growth (Call *et al*., 1995). An early synthesis of NAPS will go along well if one or more of these proteins were to act as molecular chaperones.

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Figure Legend

Fig.1. (**A**). Growth pattern of *Clostridium botulinum* type A at 37 ° and 45 °C for
different time intervals. (**B**). Effect of temperature shift-up in the exponential growth
pattern of *Clostridium botulinum* type A.

Fig.2. Induction and synthesis of 33 kDa protein at 37 ° and 45 °C in exponentially growing cells *of Clostridium botulinum* type A proteins were analyzed on 12.5 % SDS-PAGE gel under reducing conditions. Each lane of the gel was loaded with equal amounts (250 μg) of proteins. Lane 1, low molecular weight standard: Phosphorylase b, 97 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; Lane 2, cells grown at 37 °C for 0 h; Lane 3, cells grown at 37 °C for 60 min; lane 4, cells grown at 37 °C for 180 min; Lane 5, neurotoxin complex as control; Lane 6, cells grown at 45 ° C for 0 h ; Lane 7, cells grown at 45 °C for 30 min; Lane 8, cells grown at 45 °C for 60 min; Lane 9 cells grown at 45 °C for 180 min .

Fig.3. Immunoblot analysis of Hn-33 in *Clostridium botulinum* type A. (**A**). SDS-PAGE analysis of ammonium sulfate extract of neurotoxin complex of *C.botulinum* grown at 37 °C and 45 °C. Lane 1, prestained molecular weight standard in kDa: phosphorylase B, 104 kDa; bovine serum albumin, 82 kDa; ovalbumin, 48 kDa; carbonic anhydrase, 33.4 kDa; soybean trypsin inhibitor, 28.3 kDa; lysozyme, 19.9 kDa; lane 2, cells grown at normal temperature (37 °C); lane 3, cells grown at elevated temperature (45 °C); lane 4, neurotoxin complex. (**B**). Immunoblot analysis of samples in (A) with horse anti-neurotoxin complex antibodies.

A

B
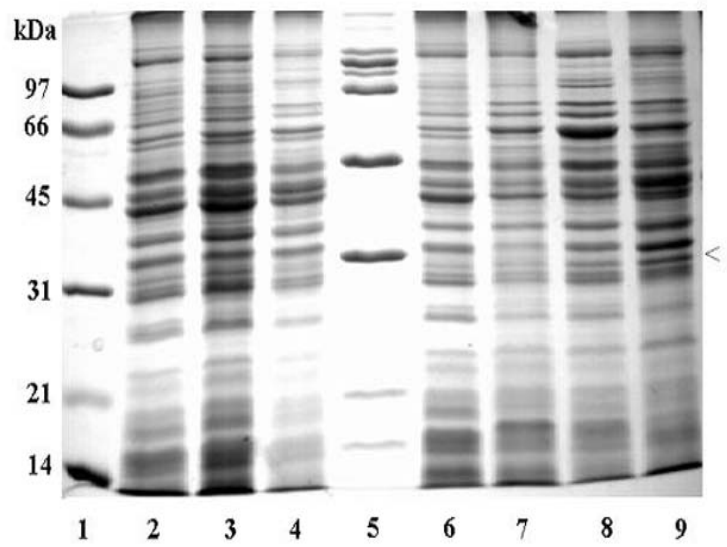
Shift-up
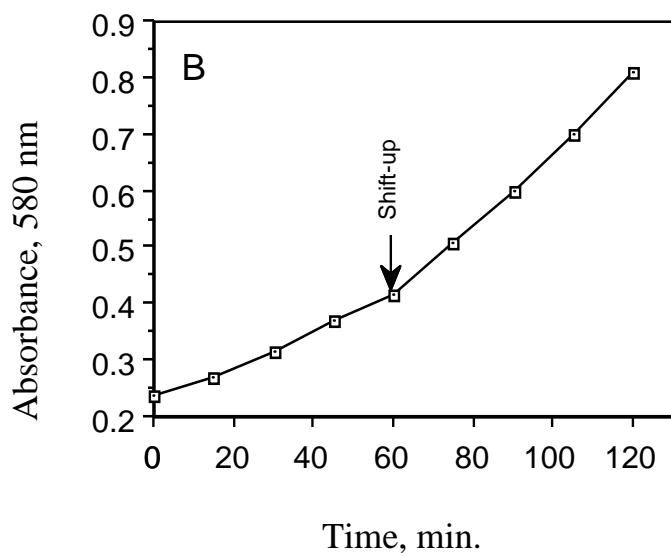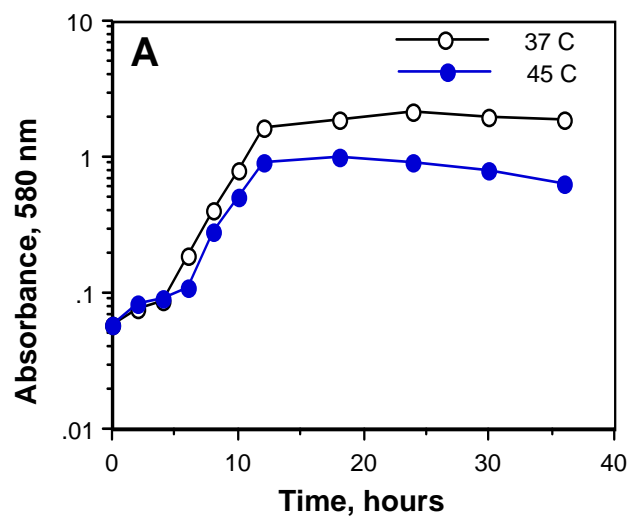
(Fig. 1 A & B)

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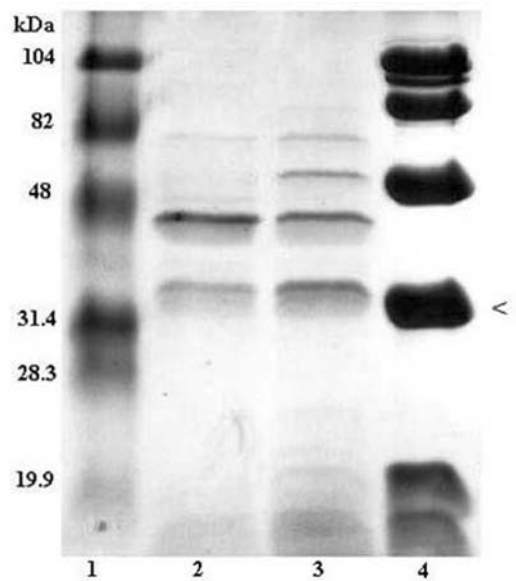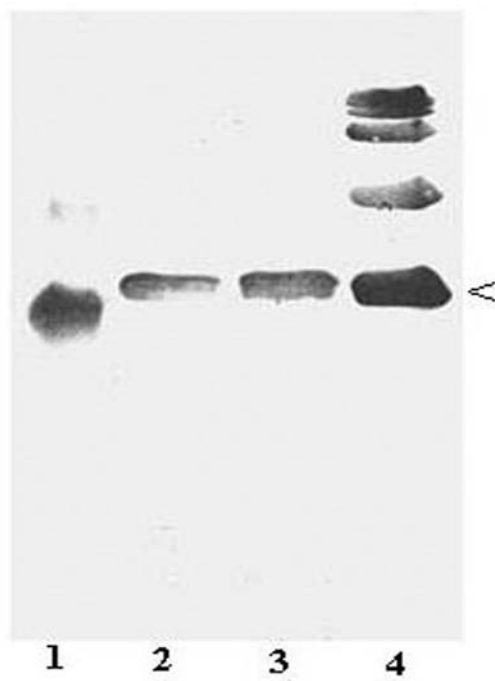
Fig. 2

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Fig. 3 A

Fig. 3B

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Microarray analysis of differentially regulated genes in human neuroblastoma and colon carcinoma cell lines upon Botulinum Neurotoxin Type A (BoNT/A) complex intoxication

Introduction

Botulinum neurotoxins (BoNTs) are the most potent natural toxins being solely responsible for the pathophysiology of botulism, one of the most terrifying paralyzing diseases to afflict humankind. (Schiavo et al., 2000). The family of BoNTs comprises of seven antigenically distinct serotypes (A to G) that are produced by various toxigenic strains of spore-forming anaerobic *Clostridium botulinum.* They act as metalloproteinases that enter peripheral cholinergic nerve terminals and cleave proteins that are crucial components of the neuroexocytosis apparatus, causing a persistent but reversible inhibition of neurotransmitter release resulting in flaccid muscle paralysis. BoNTs are produced by *C. botulinum* in the form of multimeric complexes, with a set of non-toxic neurotoxin associated proteins (NAPs) coded by genes adjacent to the neurotoxin gene. These complexes are termed as progenitor toxins (Inoue et al., 1996; Minton, 1995). A close functional relationship between BoNT and NAPs is strongly indicated by clustering of genes for BoNT, NAPs and a regulatory gene botR (Cai et al., 1999).

The most common source of botulism is by ingestion of food contaminated with spores of *C. botulinum* and preserved under anaerobic conditions that favor germination of spores and secretion of the neurotoxin (Chen et al., 1998). One of the major reasons why the toxin is active by the oral route and can be absorbed into the general circulation, is because of its association with NAPS that protect the toxin during its exposure to harsh conditions in the GI tract (Sakaguchi, 1983; Schantz and Johnson, 1992; Chen et al., 1998). NAPs are also known to assist BoNT translocation across the intestinal mucosal layer (Fujinaga et al., 1997; Fujinaga et al., 2004).The oral toxicity of BoNT increases with incremental association of the neurotoxins with NAPs (Li et al., 1998). BoNTs in their complex forms along with NAPs are known to be 10-100 fold more toxic than the non-complexed pure neurotoxin, through the oral route (Cheng et al., 2008). NAPs also have been recently shown to play a critical role in enhancing the endopeptidase activity of the neurotoxin (Sharma and Singh, 2004). Penetration of the neurotoxin through epithelial cell barriers and its subsequent migration to cholinergic nerve terminals is the first essential step of botulinum intoxication. Botulinum toxin binds to the apical surface of epithelial cells of the gastrointestinal system, undergoes receptor-mediated endocytosis and transcytosis, and is thus carried from the lumen of the gut to interstitial fluid, and ultimately to the general circulation (Maksymowych and Simpson, 1998; Maksymowych and Simpson, 2004). The role of NAPs in transport of BoNT through epithelial cells is not very clear. Some reports suggest that NAPs assist in the binding of the toxin to the epithelial cells (Fujinaga et al., 2000; Inoue et al., 2001), whereas others suggest that the ligand binding moiety is present on the neurotoxin itself (Maksmowych et al., 1999; Ahsan et al., 2005).

The clinical spectrum of botulism continues to expand including wound botulism that arises as a consequence of toxin produced in wounds contaminated with the

clostridial bacterium. Wound botulism is rare in humans and the causative agents are either type A or type B from group I, but are increasing significantly in recent years among drug users. Because of the extreme toxicity and robust stability of BoNT in the presence of NAPs and relative ease of production, BoNTs are on the top of the list of biological warfare threats (Sharma and Singh, 2004; Caya et al., 2004), and have been listed as Class A bioterror agents. Paradoxically, because of their neurospecificity, BoNTs are being exploited in the treatment of a myriad of neuromuscular disorders and for the removal of facial wrinkles (Johnson et al., 1999; Rohrich et al., 2003; Shukla and Sharma, 2005; Bigalke and Rummel, 2005; Dastoor et al., 2007; Salti and Ghersetich, 2008). Its usage against pain related disorders holds promising future therapeutics. Recent reports have suggested that there are substantial systemic adverse reactions and respiratory compromise as a result of the use of types A and B botulinum neurotoxin complex based therapeutic and cosmetic products (Botox^{TM} and Myrobloc^{TM}), leading United States Food and Drug Administration to issue medical advisory on their safety. While the exact cause of these adverse reactions are not known, inflammatory reactions involving immune system can not be ruled out at this stage.

Materials and methods

Cell culture and treatments: SH-SY5Y and HT-29 and were grown, maintained, subcultured as recommended by ATCC, using MEM/HAM-F12 (50:50) and in McCoy's 5a media respectively, supplemented with fetal calf serum to a final concentration of 10% and incubated in a 5% CO_2/humidified air at 37ºC. Cells were seeded onto 25 cm^2 culture flasks at a density of 5 x 10^5 cells per flask, after Trypsin-EDTA treatment. After 48h of seeding, the SH-SY5Y cells were treated with 6 nM BoNT/A complex in a serum free medium for 96 h. HT-29 cells were treated with 500 nM BoNT/A complex in a serum free medium for 6 h, after 24 h of seeding.

RNA preparation and Microarray analysis: The culture flasks (three independent replicates) treated or untreated with BoNT/A complex were gently washed thrice with PBS to remove the medium, and directly lysed for RNA extraction using RNeasy Mini kit, following the manufacturer's instructions (Qiagen). Whole human genome microarray was approached on the Agilent's platform. Probe labeling and hybridizations were performed at MOgene LC (St.Louis, MO, USA). Total RNA was amplified using Agilent's low input linear amplification kit as described by the manufacturer (Agilent Technologies, Santa Clara, CA, USA). About 1-5 ug of amplified target c RNA was labeled with either cy5 or cy3 using the Micromax kit (Perkin Elmer Inc., Wellesley, MA, USA) in an ozone-free enclosure. The labeled cRNA was passed through Zymo RNA Clean-up Kit (Zymo Research Corporation, Orange, CA, USA) and eluted in Nuclease free water (Ambion Inc., Austin, TX, USA). Labeled cRNA was fragmented as suggested in the Agilent processing manual. The array was scanned using Agilent scanner and data was collected using Agilent's feature software. Only genes that had at least 1.5 fold changes and passed the multiple presence tests (hit common in all three replicates) at p $\leq$ 0.01 are listed.

Results:

It is widely assumed that BoNT/A when injected for therapeutic purposes remain localised to the synapses near the injection site. However it was recently shown that BoNT/A spreads to afferent neurons via retrograde axonal transport and neuronal transcytosis to and cleaves its substrate SNAP25 (Antonucci et al., 2008), which may have serious clinical implications. This compels better understanding of BoNT physiology and trafficking in order to effectively and safely uses the botulinum neurotoxins for different therapeutic applications. This study aims to decipher the overall signaling mechanisms and global effects on gene expression during its translocation across the intestinal epithelial cells and in causing flaccid paralysis after reaching the neuromuscular junctions. We have used SH-SY5Y (human neuroblastomal) cells and HT-29 (human colon carcinoma) cells for studying the gene expression profile. SH-SY5Y cells serves as a model to study the activity of Clostridial neurotoxin serotypes (Purkiss et al., 2001), while Human colon cancer HT-29 had been used to study the internalization of *Clostridium botulinum* type C (Nishikawa et al., 2004).

The current analysis indicated that in case of HT-29 colon cancer cells, about 521 were down regulated, while about 432 seemed to be up-regulated, upon BoNT/A complex treatment for 6h. Interestingly, in the neuroblastomal SH-SY5Y cells, majority of the genes, around 455 were up-regulated, and 161 were down-regulated after 96 h exposure to the toxin A complex (with at least 1.5 fold change in relative expression). In both SH-SY5Y and HT-29 cells about 39 and 28 genes were commonly up-and down regulated (Figure 1). The selected genes were listed in Report 6&7. It was noted that enzymes involved in general metabolism were distinctly downregulated in case of HT-29 cells compared to that of the SH-SY5Y (Figs 2&3). With SH-SY5Y, genes relevant to the extracellular matrix proteins, Cell adhesion and Tight junction functions and genes relevant to inflammatory pathway were majorly upregulated (Fig. B & C). Also genes encoding Heat shock proteins and genes relevant to apoptosis were distinctly seem to be modulated in HT-29 compared to that of SH-SY5Y. Our KEGG pathway analysis in SH-SY5Y cells include the modulation of genes involved in inflammation, proteosomal degradation, phosphatidylinositol signaling pathway, calcium signaling pathway, complement and coagulation pathway and genes relevant to other neurogenerative disorders like, Huntingtons, Parkinsons and Alzheimers disease pathways. Modulation of genes relevant to tight junctions, extracellular matrix, adherens proteins and actin cytoskeleton was observed in HT-29 cells.

1) Inflammatory pathway
CCL2 chemokines - 122 fold ↑ (strongest up-regulated gene)
Matrix Metalloproteinase 9 (MMP9) - 3.9 fold ↑
**** Plasminogen activator, Urokinase (PLAU) - 2.4 fold ↑
Bradykinin receptor B1 (BDKRB1) - 46.1 ↑
 *** Bradykinin receptor B2 (BDKRB2) - 5.9 fold ↑
IL-32 - 36.8 fold ↑

2) Proteosomal degradative pathway
Proteasome beta subunit 8 (PSMB8) - 2.6 fold ↑
Beta type, 9 (large multifunctional protease 2) (PSMB9) - 1.92 fold ↑
Proteasome subunit alpha type 7 (PSA7, RC6-1) – 3.7 ↑
**Ubiquitin-like, PHD and RING finger domains, 1 (UHRF1) - 2.0 fold ↓
Ubiquitin specific protease 28 (USP28) - 1.58 fold ↓
Ubiquitin specific peptidase 40 (USP40) - 1.0 fold ↑

3) Phosphatidylinositol signaling pathway
Synaptojanin 2 (SYNJ2) - 1.9 fold ↑
***Bradykinin receptor B2 (BDKRB2) - 5.9 fold ↑
Inositol polyphosphate-5-phosphatase, 145kDa (INPP5D) - 5.9 fold ↑
Mitogen-activated protein kinase kinase 6 (MAP2K6) - 1.7 fold ↓

4) Complement and Coagulation pathway
****Plasminogen activator, Urokinase (PLAU) – 2.4 fold ↑
Complement component 1, r subcomponent (C1R) - 5.8 fold ↑
Complement component 1, s subcomponent (C1S) - 9.0 fold ↑
B-factor, properdin (BF) - 17.6 fold ↑
Complement component 3 (C3) – 7.2 fold ↑

5) Calcium signaling pathway
 *** Bradykinin receptor B2 (BDKRB2) BDKRB2) - 5.9 fold ↑
Peptidylprolyl isomerase C (Cyclophilin C) (PPIC) - 2.0 fold ↓

6) Huntington disease
 ** Ubiquitin-like, PHD and RING finger domains, 1 (UHRF1) - 2.0 fold ↓
Transglutaminase 2 (C polypeptide) (TGM2) - 3.4 fold ↑

7) Alzheimers disease
Enkephalinase membrane metallo-endopeptidase (CALLA) - 2.3 fold ↑

8) Parkinsons disease
Ubiquitin-like, PHD and RING finger domains, 1 (UHRF1) - 2.0 fold ↓

9) GnRH signaling pathway
Phospholipase D1, phophatidylcholine-specific (PLD1) – 4.0 fold ↑
 *** Bradykinin receptor B2 (BDKRB2) BDKRB2) - 5.9 fold ↑

10) Long term potentiation/Depression
 *** Bradykinin receptor B2 (BDKRB2) BDKRB2) - 5.9 fold ↑

11) Others
Ubiquitin D (UBD) (Aliases - Diubiquitin, FAT10) - 98.5 fold ↑
Neuronal specific transcription factor DAT1 (DAT1) - 2.3 ↓

A

1) Focal adhesion, Cell adhesion molecules (CAMs), Adherens junction, and Gap junction related Pathways
Plasminogen activator - 1.3 fold ↑
Proprotein convertase subtilisin/kexin type 9 (PCSK9) -   2.1 fold ↓
Phosphatase and tensin homolog (PTEN) - 1.4 fold ↑
Dual specificity phosphatase 6 (DUSP6) - 1.6 fold ↓
Histone acetyl transferase (MYST4) - 1.4 ↑
Gap junction protein beta, 5 (Connexin 31.1) - 5.0 ↓
Phospholipase C (PLC) - 3.0 fold ↑
Intercellular adhesion molecule 4 & 5 (ICAM4 & ICAM5) - 2.4 & 2.3 fold ↓

2) **Regulation of Actin cytoskeleton**
Phosphatidylinositol-4-phosphate 5-kinase, type I, beta (PIP5K1B) - 2.4 fold ↑
Phosphoinositide-3-kinase, catalytic, delta polypeptide (PIK3CD) - 2.5 fold ↓

3) Metabolism of xenobiotics by cytochrome P450
UDP glycosyltransferase 2 family, B15, (UGT2B15) - 2.0 fold ↓
Leukotriene C4 synthase (LTC4S) - 2.1 fold ↓

4) Others
Calcium channel, gamma subunit 8 (CACNG8) & P/Q type, alpha 1A subunit (CACNA1A) - 2.4 fold & 4.4 fold ↓
Collagen, type IX, alpha 1 (COL9A1) - 2.3 fold ↓
Caveolin 1 & 2 (CAV1 & CAV2) - 2.3 & 2.1 fold ↓
E3 ubiquitin protein ligase 2 (WWP2) - 3.1 fold ↑

B

Fig.1. KEGG pathway analysis of the statistically significant up-regulated (indicated as ↑) and down-regulated genes (indicated as ↓) in SH-SY5Y cells (A) and HT-29 cells (B) upon exposure to the BoNT/A complex. ** Gene implicated in Parkinson's disease pathway; ***Gene implicated in Gap junction, VEGF, Wnt, Natural Killer Cell mediated Cytotoxicity, Fc epsilon RI signaling, Leukocyte transendothelial migration associated pathways; ****Gene implicated in Neuroactive ligand-receptor interaction, Cell communication, Focal adhesion associated pathways

Discussion

This study aim to decipher the overall signaling mechanisms and global effects on gene expression in Human colon cancer HT-29 and neuroblastomal SH-SY5Y cell lines that are ideal model for studying BoNT physiology. The HT-29 and SH-SY5Y cells were treated with 500nM BoNT/A complex (serum free medium) for 6h, and 6nM BoNT/A complex (serum free medium) for 96h, respectively. The changes in the gene expression

profile and magnitude of response in gene expression in those cells could be attributed to the differences in the receptor mediated entry mechanisms upon transcytosis and endocytosis.

Genes relevant to neuroinflammation: Botulinum neurotoxins effects on CNS upon direct penetration of Blood-Brain Barrier (BBB) are still not very well known. Although in mice models they were reported to accumulate in brain (Boroff DA and Chen GS., 1975), it is believed that their entry across BBB could be restricted due to its size (150kDa), and also by the fact that they do not reach significant systemic levels upon intramuscular, subcutaneous therapeutic injections (Cui et al., 2004). However recent reports show that they could reach CNS by retrograde axonal transport and neuronal transcytosis (Dressler and Saberi., 2005; Antonucci et al., 2008). The array results indicate up-regulation of genes involving matrix metalloproteinase-9 (MMP-9); plasminogen activator (urokinase) which is involved in the activation cascade of MMPs (Ref), and chemokines like CCL2, CXCL12. MMPs are reported to attack the extracellular matrix proteins in the basal lamina, such as fibronectin, laminin, and collagen type IV, increasing the permeability of the blood-brain barrier. MMP-9 up-regulation has been observed in several neuropathological conditions that compromise the BBB integrity in multiple sclerosis (Birkedal-Hansen et al., 1993), in viral infections (Wang et al., 2004). CCL2, CXCL12 are known to play key role in transmigration of monocytes and T-cells across BBB (Ge et al., 2008). The internalization of BoNTs in peripheral leucocytes is not known, although it has been shown in non-neuronal cells like astrocytes of brain (Verderio et al., 1999) that are potent source of inducible MMP-9 and CCL2 (Rosenberg., 2005). These observations suggests careful clinical validation in extending the application of BoNTs to several CNS disorders associated with neuronal exocytosis (Verderio et al., 2007), since they may alter the MMPs in brain and recruit the effectors of inflammation in CNS.

Genes relevant to Ubiquitin-Proteosomal degradation: Interestingly, it was observed that many genes related to ubiquitin-proteasomal degradative pathway. Some of the component subunits of the proteasome machinery like beta subunit 8 (PSMB8), and Proteasome subunit alpha type 7 (PSA7, RC6-1), were upregulated at the transcriptional level. Gene encoding Ubiquitin-like, PHD and RING finger domain, 1 (UHRF1) and Ubiquitin specific protease 28 (USP28), a deubiquitinating enzyme (DUBs) was down-regulated. It was also noted that a Beta type, 9 (large multifunctional protease 2) (PSMB9) was up-regulated 1.9 fold, but was found only in two sets of the triplicate RNA sample used for the microarray. Similarly, ubiquitin specific peptidase 40 (USP40) was observed to be 1.0 fold up-regulated. It is becoming increasingly recognized to include such genes in the data set if they are relevant to a pathway which is significantly altered, in order to exclude indiscriminate exclusion of those genes by multiple presence test and by fold difference (Duke et al., 2006). It is noteworthy that overexpression of PSA7 or RC6-1 impair late endocytic transport and lysosomal transport (Dong et al., 2004) and also the role of DUBs in several neurodegenerative disorders. Failure of the ubiquitin-proteasome system (UPS) that play an important role in the pathogenesis of Parkinson's disease had been earlier to differentially express the genes encoding UPS 26S and 20S proteasomal

subunits and E1, E2 and E3 enzymes. UHRF1 is up-regulated in Parkinson's whereas we see down-regulation in case of botulinum intoxication.

Genes/pathways relevant to HT-29: Genes involved in the adherens and tight junctions, were observed in HT-29 cells. It has recently reported that the Haemagglutinin (HA) protein of botulinum toxin disrupts the structure and function of the epithelial intercellular tight and adherens junctions (Matsumura et al., 2008). The modulation of genes associated with adherens and tight junctions could be manifestation of the HA activity or as an adaptive response of the cells against the HA activity.

Our results would be physiologically more relevant to wound borne botulism and therapeutic applications of botulinum neurotoxin, and emphasize the necessity to test their therapeutic consequences by clinical validation in patients in the light of recently reported adversities.
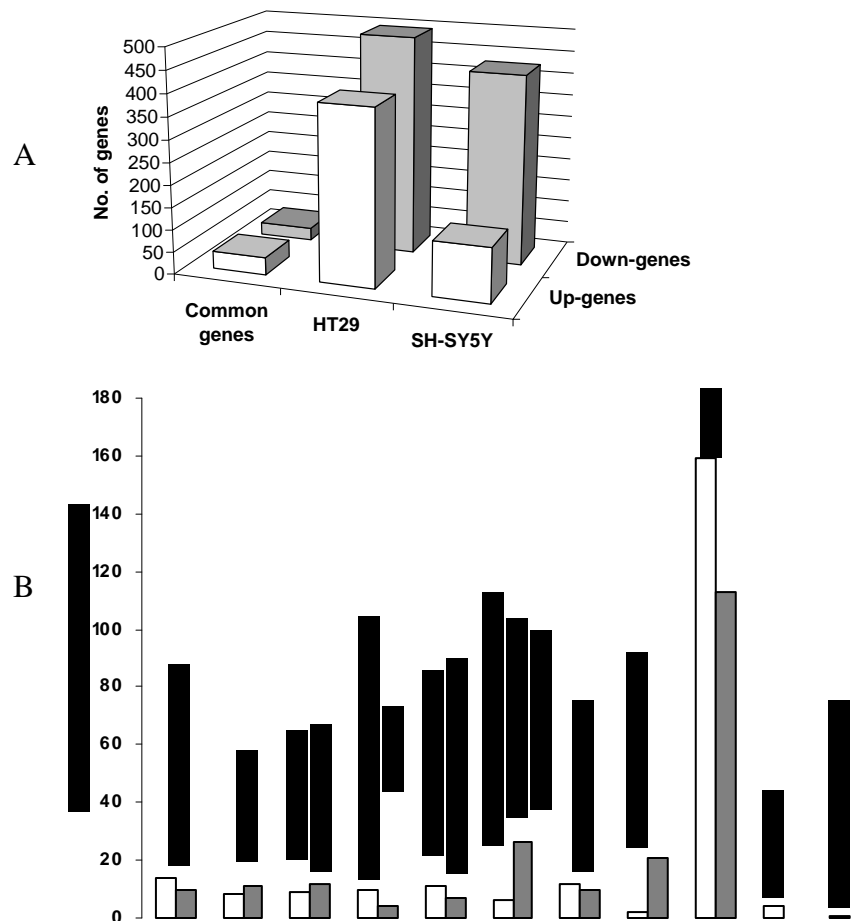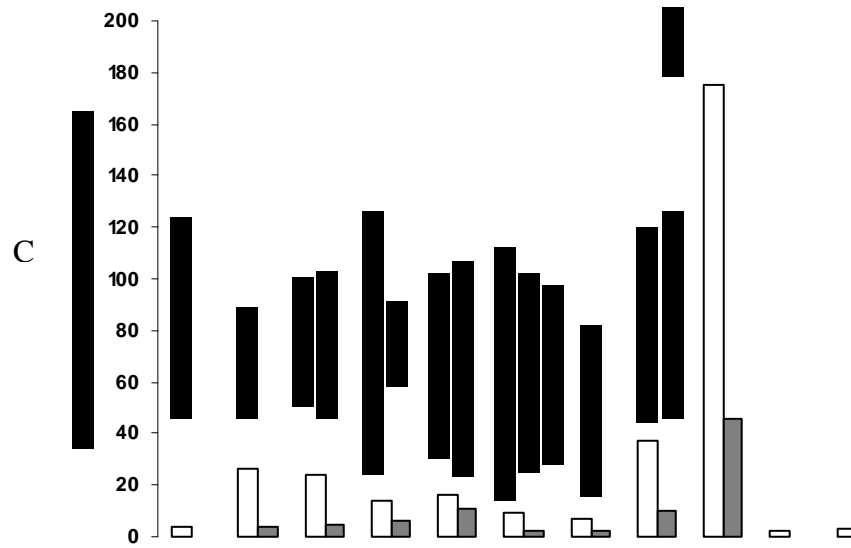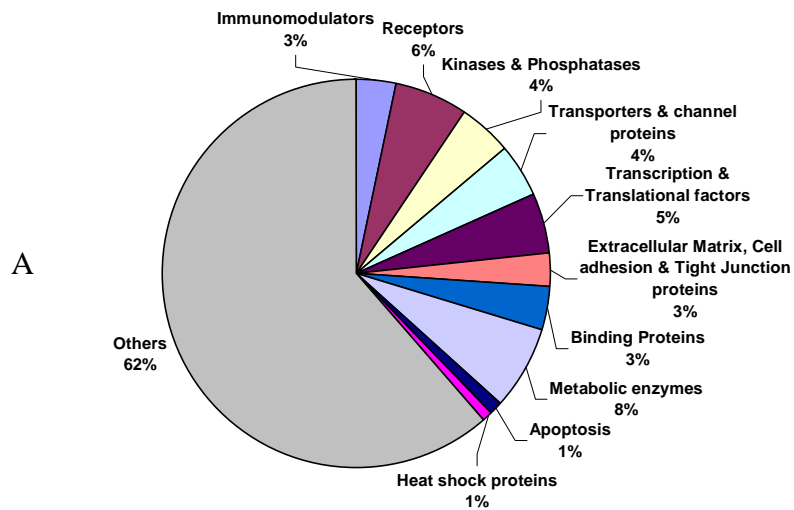
Fig. 2. Expression pattern of functionally classified genes in HT-29 and SH-SY5Y upon BoNT/A complex intoxication (A). Functional distribution of genes that are Up- and Down-regualted genes in HT-29 (A), and SH-SY5Y (B).
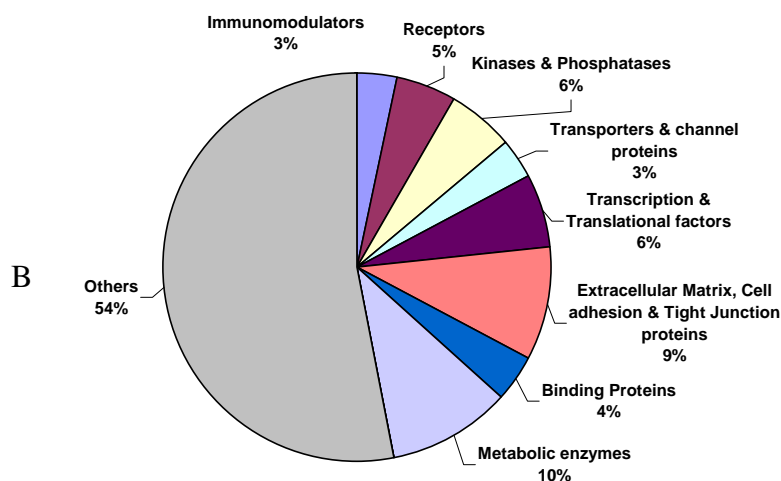
Fig. 3. Pie charts showing the functional distribution of genes that are modulated in HT-29 (A), and SH-SY5Y (B).

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